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Title	Optimisation of protein and lipid extraction from low-value fish sources and investigation of high-value applications
Authors	Egerton, Sian
Publication date	2019-07
Original Citation	Egerton, S. 2019. Optimisation of protein and lipid extraction from low-value fish sources and investigation of high-value applications. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2023-05-05 23:30:28
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Optimisation of protein and lipid extraction from low-value fish sources and investigation of high-value applications

Thesis presented to the National University of Ireland, Cork
for the degree of Doctor of Philosophy
by

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July 2019

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Microbiome Ireland
Interfacing Food & Medicine
IRISH RESEARCH COUNCIL
An Chomhairle um Thaighde in Éirinn
teagasc
Agriculture and Food Development Authority

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Declaration

This thesis has not been previously submitted to this or any other university for any degree and is, unless otherwise stated, the original work of the author.

Author Contribution

All of the work herein was performed by the author with the following exceptions:

Chapter 4.

Dr Fiona Fouhy performed part of the bioinformatics analysis of 16S sequencing data including QIIME processing. Dr Stephen Stockdale performed the bioinformatics analysis of viral DNA shotgun sequencing and produced the relating figures of the results.

Chapter 6.

Dr Kiera Murphy performed part of the bioinformatics analysis of 16S sequencing data including QIIME processing. Dr Alex Wan and Mr Niall Muller performed the gut histology analysis. Dr Alex Wan, Dr Fergus Collins, Dr Kizkitka Busca, Dr Fintan Egan, Ms Grace Ahern, Mr Ivan Sugrue and Mr Niall Muller assisted with the sample collection.

Chapter 7.

Mr Francisco Donoso conducted the behavioural tests, monoamine detection and relating analysis. Ms Sian Egerton assisted Mr Francisco Donoso in conducting the plasma corticosterone and BDNF analysis.

Publications

THESIS PUBLICATIONS

- **Egerton, S.,** Culloty, S., Whooley, J., Stanton, C., Ross, R.P. and Handling editor: Emory Anderson, 2017. Boarfish (*Capros aper*): review of a new capture fishery and its valorisation potential. *ICES Journal of Marine Science*, **74**(8), pp.2059-2068.

- **Egerton, S.,** Culloty, S., Whooley, J., Stanton, C. and Ross, R.P., 2018. The gut microbiota of marine fish. *Frontiers in Microbiology*, **9**.

- **Egerton, S.,** Culloty, S., Whooley, J., Stanton, C. and Ross, R.P., 2018. Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification. *Food Chemistry*, **245**, pp.698-706.

- **Egerton, S.**, Wan, A., Murphy, K., Collins, F., Ahern, G., Sugrue, I., Busca, K., Egan, F., Muller, N., Whooley, J., McGinnity, P., Culloty, S., Ross, P., Stanton, C. Replacing fishmeal with plant protein in Atlantic salmon (*Salmo salar*) diets by supplementation with fish protein hydrolysate. *Scientific Reports, accepted January 2020*.

ABBREVIATIONS

AA – Amino acids

ACE – Angiotensin -converting enzyme

ALA – Alpha-linolenic acid

AUC – Area under the curve

BCAA – Branched chain amino acids

BCFA – Branched chain fatty acids

BDNF – Brain-derived neurotrophic factor

BF - Boarfish

BII – Biomarine Ingredients Ireland (Ltd.; thesis enterprise partner)

BW – Blue whiting

CFU – Colony forming unit

CON - Control

DA – Dopamine

DAFM – Department of Agriculture, Farming and the Marine

DHA – Docosahexaenoic acid

DOPAC – 3,4-Dihydroxyphenylacetic acid (dopamine metabolite)

EAA – Essential amino acids

EPA – Eicosapentaenoic acid

EFSA – European Food Safety Authority

ELS – Early life stress

FAA – Free amino acids

FAME – Fatty acid methyl esters

FAO – Food and Agriculture Organisation (of the UN)

FDR – False discovery rate

FFA – Free fatty acids

FO – Fish oil

FPH – Fish protein hydrolysates

FX - Fluoxetine

GABA – Gamma aminobutyric acid

GC – Gas chromatography

GIT – Gastro-intestinal tract

GL – Glycolipids

5-HIAA – 5-hydroxyindoleacetic acid (serotonin metabolite)

HPA – Hypothalamus-Pituitary-Adrenal

HPLC – High performance liquid chromatography

HR – Herring

5-HT – Serotonin

IRC – Irish Research Council

LA – Linoleic acid

LTL – Lower trophic level

MAL – Maximum allowable limit

MAO – Monoamine oxidase

MDD – Major depressive disorder

MS – Maternally separated

MUFA – Mono-unsaturated fatty acids

N-3 – Omega 3

N-6 – Omega 6

NA – Noradrenaline

NS – Non-separated

PCR – Polymerase chain reaction

PHP – Partly hydrolysed protein

PND – Post-natal day

SCFA – Short chain fatty acids

SPH – Soluble protein hydrolysate

SSRI – Selective serotonin reuptake inhibitor

OTU – Operational taxonomic unit

PHP – Partly hydrolysed protein (BII commercial FPH)

PL -Phospholipids

PUFA – Poly-unsaturated fatty acids

SFA – Saturated fatty acids

SPH – Soluble protein hydrolysate (BII commercial FPH)

TG – Triglycerides

UN – United Nations

WESPAS – Western European Shelf Pelagic Acoustic Survey

WHO – World Health Organisation

WPH – Whey protein hydrolysate

Thesis Abstract

ABSTRACT

The aim of this thesis was to investigate potential high value nutritional applications for protein and lipid raw material extracted from low-value pelagic marine fish, specifically blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*), and Atlantic herring (*Clupea harengus*), and to assess the effects of such products on the recipient's gut microbiota.

In Chapters 3 and 4 the proximate (biomass in terms of gross components, e.g. water, ash, protein and lipids) and gut microbial compositions of the three fish species were studied. Boarfish had the highest mineral (ash) content (6%), herring had the largest lipid fraction (11%) while protein level was similar in all three species (16 – 17%). Interestingly, the composition of the gut microbiome of the three fish were significantly different. The dominating phylum was Proteobacteria, followed by Tenericutes and Spirochaetes in all species, however, seven phyla were only recorded in one fish species and only two bacterial families (*Vibrionaceae* and *Lactobacillaceae*) were detected in the core microbiota of all three fish. Unique and novel viromes that separate distinctly in relation to fish species, and diverge from ocean and human viromes, were also found.

Subsequent chapters in the thesis concentrate on blue whiting only. Chapter 5 and 6 focus on the protein fraction of the fish. Firstly, protein hydrolysates were produced using a range of enzymes. The resulting protein powders showed antioxidant properties and high solubility (> 80%) across a wide pH range in water. Moreover, their solubility improved further within a vitamin-tea beverage matrix (> 85%), proving them an ideal functional food for beverage fortification. In Chapter 6, two commercially-produced fish protein hydrolysate powders (1. powder of the soluble protein hydrolysate (SPH) fraction; 96% soluble protein, and, 2. powder of the partly-hydrolysed protein (PHP) fraction; 18% soluble protein) were used to supplement salmon aquafeeds high in plant protein. The growth performance of salmon fed the partly-hydrolysed protein hydrolysate powder equalled that of fish fed the positive control 35% fishmeal diet. Fish on diets supplemented with protein hydrolysates had significantly higher levels of many amino acids in their blood, including 27 – 48% more branched chain amino acids compared to fish fed the 35% fishmeal diet. Replacement of fishmeal with high levels of plant protein significantly altered the salmon gut microbiota. Alpha diversity was reduced and Spirochaetes as well as the families *Moritellaceae, Psychromonadaceae, Helicobacteraceae* and *Bacteroidaceae* were all found at significantly

lower abundances compared to the control fishmeal diet. Supplementation with the SPH hydrolysate reduced the effects of high dietary plant protein on the gut microbiota.

Finally, the last chapter of the thesis investigates the efficacy of fish oil dietary supplementation, on its own and in conjunction with the anti-depressant fluoxetine, as a treatment for depressive and anxiety-like symptoms associated with early life stress. The maternal separation model of early life stress was used in male Sprague-Dawley rats to test these treatments. Maternally separated rats showed depressive-like behaviours in the forced swim and open field tests. Depressive-like behaviour in the forced swim test was evaded by treatment with fluoxetine only. Anxiety-like behaviours in the open field test were not observed in any of the three treatment groups (fish oil, fluoxetine, fish oil + fluoxetine). Maternally-separated rats fed the fish oil, fluoxetine and fish oil + fluoxetine diets all had significantly lower plasma corticosterone levels and brain stem monoamine turnover was significantly changed compared to the untreated maternally separated rats. Fish oil significantly reduced caecal levels of butyrate while fluoxetine increased both acetate and propionate levels. Correlating changes were recorded in the SCFA-producing gut microbiota. Fish oil treatment significantly altered biological markers of stress and depression as well as the gut microbiota which translated to an evasion of anxiety-like behaviour.

Taken together, the studies here within demonstrate that these three species of fish may provide a rich and valued source of ingredients for a variety of applications in animal, fish and human foodstuffs.

Chapter 1

General Introduction

GENERAL INTRODUCTION

There is a growing need and desire to increase sustainable practices in the marine sector. Wild fisheries and the seafood industry are areas in which this has reached a critical stage, with the EU implementing a ban on discarding unwanted catch in a phased operation between 2014 and 2019. Fisheries resources are limited, and the consensus opinion is that all catches should be landed and utilised. In theory, such a policy is needed with respect to food security and sustainability. However, putting the policy into practice will require adaptation of markets and the processing sector to accommodate a wider range of catch components (Borges *et al.* 2016).

New advanced processing technologies provide the opportunities to develop new uses for fish catches that may increase value of some lower value species (often previously discarded). The recovery of chemical components from low-value fish and waste by-products, which can be used in food, cosmetics and pharmaceuticals, is a promising area that could add significant value to fishers' landings. In order to develop such valorisation strategies, it is firstly imperative that baseline knowledge of the composition of the raw material is established. Secondly, new approaches and optimization of processing methods will need to be established. When high quality products are produced from the raw material, appropriate uses will need to be investigated before markets and sales can be developed. The studies in this PhD thesis aimed to address all the steps outlined prior to market development, with a specific focus on investigating the nutritional benefits of these products and their effects on the recipient's gut microbiota.

While progress and development of the fisheries sector should be supported, it is important to consider the associated environmental and ecological impacts. With increased interest in lower trophic level (LTL) species, known as 'fishing down the food chain', there are concerns of possible trophic cascades, negative impacts on ecosystem structures and reductions in ecosystem and community resilience (Maureaud *et al*., 2017; Tromeur and Loeuille, 2018). To minimise impact and increase sustainability, it is important that the industry develops within an ecosystem-based fisheries management system (Trochta *et al.,* 2018) and focusses on reducing waste and maximising profitability from the fish caught.

The focus of this research began with three low value pelagic fish species; blue whiting (*Micromesistius poutassou*), Atlantic herring (*Clupea harengus*) and boarfish (*Capros aper*).

Blue whiting is caught in some of the greatest numbers in the Northeast Atlantic. A rise in stock numbers in the 1990's led to European catch sizes growing by 500% (Payne *et al.* 2012). In 2004, the Northeast Atlantic blue whiting fishery was the third largest in the world (Pointin and Payne 2014). Blue whiting has become one of the most important raw materials for fish oil and fishmeal (Alder *et al.* 2008). However, its value remains low as there is little or no demand for human consumption.

Atlantic herring is one of the top ten species contributing to global catches (UN 2010). The North Atlantic herring stock is large and complex. It has been an important species in many countries, including Ireland. However, historical fluctuations in landings have added to the challenge of changing market trends for this species. The Irish herring fishery initially grew from a Europe-wide demand for fresh and processed herring but as tastes have changed in the previous decades, these fish are now also commonly sold at low prices for reduction to fishmeal (Neiland 2007).

Boarfish is a relatively new fishery (reviewed in detail in Chapter 2.1), with the first ICES recorded landings in 2001 (Nolan 2014). Landings remained low until after 2006 when they started to increase exponentially with a high of 207,882 tonnes landed in 2012. Ireland has led the development of this fishery and receives the bulk of the quota. A number of European countries, especially Ireland and Spain, have investigated possible product and market options for this fishery but this has partly been hampered by the challenges with processing these bony fish and also by the fluctuating catch volumes. Therefore, the majority of boarfish caught to date have been used for fishmeal production and other low value streams.

Even though these fish species make up a major component of the Irish fishing industry in terms of tonnage, they currently do not contribute considerably to the value of landings (Gerritsen and Lordan 2014). As is the case for many nations, Ireland's annual total allowable catches (TACs) and quota allocations have been decreasing over the past decade in response to growing concerns about the overexploitation of numerous fish stocks (Paulrud *et al.* 2014). These are important regulations required to help protect fish stocks for future exploitations and wider ecological integrity. Therefore, if Ireland wants to increase the profitability of its fisheries sector while complying with scientific advice of minimum sustainable yield, it will need to look at innovative and sustainable ways of increasing the

value, rather than the volume, of landings, especially in the pelagic fisheries (Egerton *et al.* 2017).

The global health and wellness market is estimated to be worth 4.2 trillion USD (Yeung and Johnston, 2018). Nutraceuticals are a growing part of this market. Sometimes referred to as functional foods, they are natural bioactive, chemical compounds that have health-promoting, disease-preventing, or medicinal properties (DeFelice, 1994). Some peptides (short chain proteins) and certain fatty acids within fish oils (particularly omega-3 polyunsaturated fatty acids) can act as nutraceuticals providing health benefits through numerous biological pathways.

In the last decade the role of the gut microbiota plays in all aspects of the body's systems has come to light (Zhao and Shen, 2010). The gut microbiota act as key elements in the body that facilitate the exploitation and conversion of dietary nutrients into active compounds to be used for host growth, development and maintenance (Cummings and Macfarlane, 1997; Oriach *et al.*, 2016). Inclusion of the gut microbiota in the study of nutrition and host health have hence become imperative.

The studies here within aim to contribute to the investigation and development of high-value nutritional applications for protein and lipid fractions from low-value fish sources and the assessment of their nutritional qualities, especially in relation to consumer's gut microbiota. I began by reviewing the potential for developing such applications from a low-value pelagic fishery, using boarfish as an example (Chapter 2.1). Following this, current knowledge of fish gut microbiota is reviewed in preparation for subsequent studies (Chapter 2.2). Chapter 3 and 4 provide baseline knowledge of the microbial and proximate compositional (biomass in terms of gross components, e.g. water, ash, protein and lipids) make-up of the three fish species of interest; blue whiting, herring and boarfish. Subsequently, investigations of possible applications for fish protein (Chapter 5 and 6) and oil extracts (Chapter 7) are outlined in the three ensuing chapters.

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Chapter 2.1

Boarfish (*Capros aper*): review of a new capture fishery and its valorisation potential

Published: *ICES Journal of Marine Science* (2017), **74**(8), 2059-2068.

doi.org/10.1093/icesjms/fsx048

ABSTRACT

The world's fish stocks, although renewable, are a finite resource. European capture fisheries have remained stagnant in terms of volume for many years. To remain profitable, fishers are looking for new opportunities to diversify, reduce costs, and maximize profits. The targeted fishing of boarfish (*Capros aper*) in Europe is an excellent example of such adaptation. Using this fishery as a case study, we highlight how established fisheries are adapting to changes faced by the industry. We begin by compiling the knowledge to date on the taxonomy, biology, and ecology of the understudied boarfish and go on to provide a comprehensive overview of its expansion as a targeted fishery in Europe, examining the range of valorisation options currently being investigated.

1. INTRODCUTION

Global fish consumption has been growing at an average annual rate of 3.2% since the early 1960s (FAO 2016). Demand is set to continue, driven by a rising population, food preference and a growing recognition of the importance of fish as a key ingredient for health and nutrition. As demand increases, so do constraints on the supply side of the chain. European capture fisheries have remained stagnant for many years, constrained by restrictive management policies and lowered quotas, implemented in an attempt to revive depleted stocks (Abernethy *et al.* 2010). Fishers and fishing communities face profound environmental challenges such as declines in marine resources and climate change (Cheung *et al.* 2012), as well as economic pressures with rising fuel costs, and an increasingly global trade in fish (Cheilari *et al.* 2013). These changes, the intrinsic uncertainty associated with fishing, combined with increasing regulations, cumulate and are testing the resilience of fishing communities. However, these challenges are also encouraging innovation and reformation within the industry. Fishers are changing their behaviour to cut costs, reduce waste, maximize usage, and increase quality and profitability, e.g. by reducing fuel consumption and improving handling and storage of fish on board. They are also looking for new sources of opportunity for income and in this regard, the understudied species *Capros aper* (boarfish) provides an excellent opportunity. However, the targeted fishing of boarfish was only established in the last 15 years, with concerted efforts and an expansion seen in the last 10 years. For this reason, little interest has been given to this species until recently and only a sparse amount of literature relating to it has been published (White *et al.* 2010; Stange 2016).

In this review, we compile the knowledge to date on the taxonomy, biology, and ecology of boarfish – information that is greatly sought after as organizations hasten to implement accurate and appropriate quotas for a nascent fishery. In addition, we provide a comprehensive overview of its expansion as a targeted fishery in Europe. The Irish boarfish fishery is used here as a case study to highlight how established fisheries are adapting to changes faced by the capture fishing industry around the world. In addition, future opportunities for valorisation, from human consumption to bioprocessing opportunities, are discussed.

2. BOARFISH, THE SPECIES

2.1 Taxonomy

The boarfish (*Capros aper,* Linnaeus) is the only species in its genus, belonging to the family *Caproidae* and suborder Caproidei. It is of the order Perciformes, the largest order of vertebrates which includes over 10 000 species of bony fish (Nelson 2006). There have been suggestions, however, that the suborder Caproidei should in fact be considered as pre-perciform (Rosen 1984; Tyler *et al.* 2003). There are others who propose that the family *Caproidae* be placed under the order Zeiformes (Yamanoue *et al.* 2007), and disagreement on the relationship between Caproids, Tetraodontiformes, and Zeiformes continues today (Bieńkowska-Wasiluk and Bonde 2015). This reflects the unsettled classification of the Perciforme order, which is expected to shift as new evidence emerges (Nelson 2006).

The suborder Caproidei consists of 18 species (Figure 2.1.1.; Yapıcı and Filiz 2014). *C. aper's* closest relatives are in the sister genus *Antigonia* and include species like the deepbody boarfish (*Antigonia capros*), which is widely distributed throughout tropical and sub-tropical oceans, and the shortspine boarfish (*Antigonia combatia*), found in the Western Atlantic.

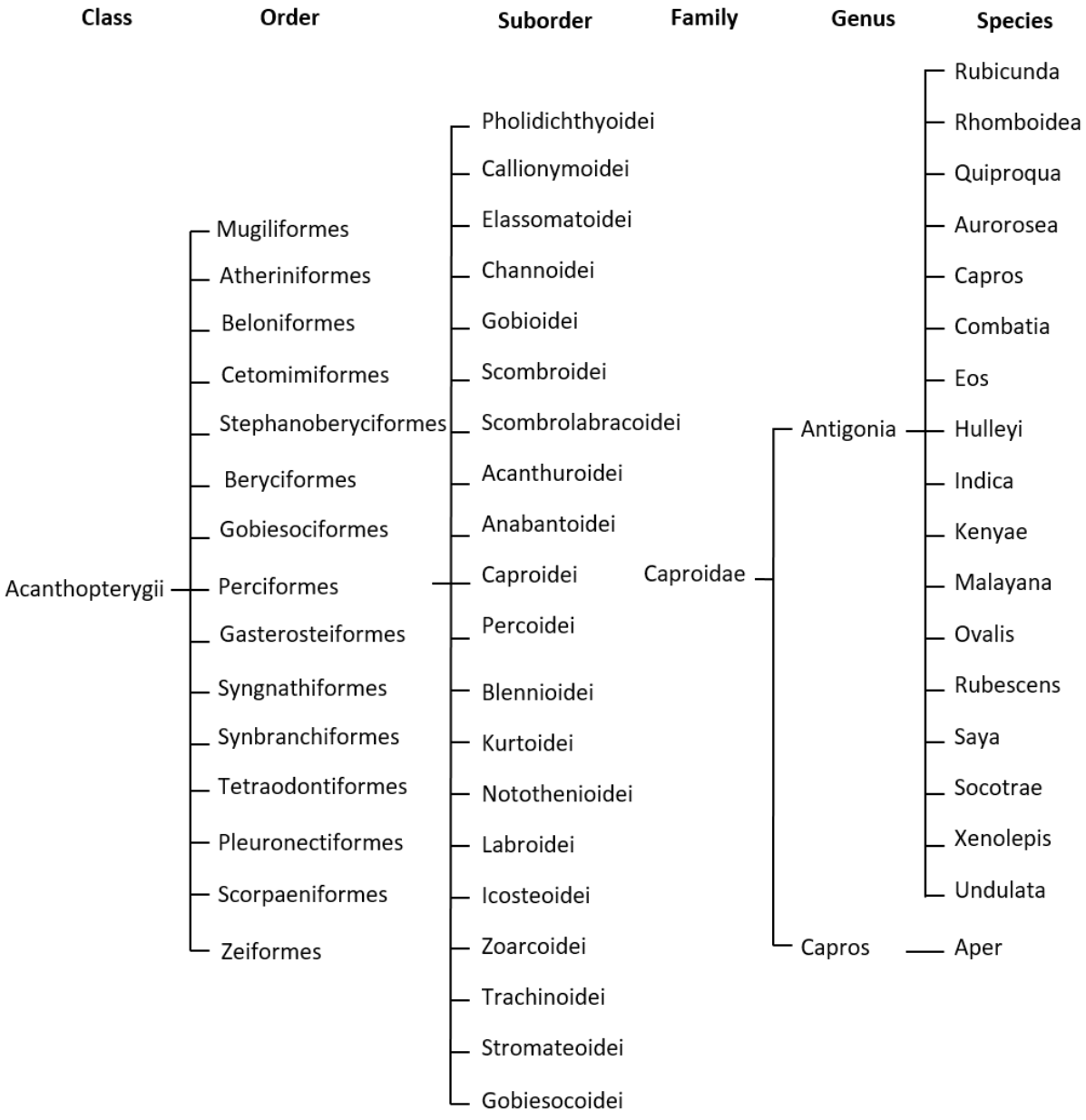

Figure 2.1.1. Phylogenetic tree outlining how *Capros aper* is related within the class of Acanthopterygii fish.

2.2 Biology

Boarfish resemble the shape of a small John Dory (*Zeus faber*). They are small, deep-bodied, laterally compressed, pelagic, shoaling fish (White *et al.* 2010; Nolan 2014). They are orange to red in colour, with ctenoid scales, robust dorsal and pectoral spines, and a small protractible mouth (Figure 2.1.2.; Nelson 2006 ; White *et al.* 2010; Yapıcı and Filiz 2014). Previously little was known about the biology and life history traits of *C. aper.* Over the past decade, however, a growing number of studies have published papers on this topic, contributing to an increase in knowledge of this species. Data, thus far, have mainly been collected in the Northeast Atlantic and the Aegean and Mediterranean Seas. In the Northeast Atlantic, the maximum size recorded for boarfish is 23 cm total length (TL), but they average 9 – 10 cm (White *et al.* 2010; Nolan 2014). In this region, length- and age-at-maturity were calculated at 9.72 cm TL and 3.4 years, respectively (Hussy *et al.* 2012). These fish are estimated to live up to 30 years (Hussy *et al.* 2012) and are considered sexually dimorphic, with larger females. Additionally, two separate morphotypes were observed off the coast of Portugal (Lopes *et al.* 2006). Studies in the Aegean and Mediterranean Seas found no such dimorphism (Yapıcı and Filiz 2014). Other differences between boarfish observed in this region include size, age, and length- and age-at-maturity. The largest recorded individual in the study in the Aegean Sea was 10.5 cm TL and the oldest specimens were 4 years; similar observations were reported in the Mediterranean Sea (Kaya and Özaydin 1996; Yapıcı and Filiz 2014). These studies estimated length- and age-at-maturity were reached at 6.69 cm TL and 1.84 years and 8.5 cm TL and 2 years, respectively (Kaya and Özaydin 1996; Yapıcı and Filiz 2014). More data of this type are critically needed to confirm the (non-) existence of subpopulations within this species so that it can be appropriately managed and conserved.

The growth pattern of *C. aper* indicates that they reach an asymptotic size relatively quickly and thereafter can reallocate energy towards reproduction (White *et al.* 2011). In the Northeast Atlantic, spawning aggregations form off the southwest coast of Ireland (Blanchard and Vandermeirsch 2005). They are income spawners, taking in energy during this period, which starts between February and April and finishes between October and December (Farrell *et al.* 2012; Nolan 2014). With indeterminate fecundity, the physiological capability to spawn repeatedly, longevity with continued reproduction at old age, and relatively young age at maturation, boarfish are likely to be a highly productive species that could take advantage of

favourable conditions to increase in abundance (Farrell *et al.* 2012; Hussy *et al.* 2012). Further studies that collect data on their spawning behaviour will help to prove this hypothesis and define their productivity as well as their ability to withstand fishing pressure.

Figure 2.1.2. Boarfish (*Capros aper*). Photo ©: E. Farrell.

2.3 Ecology

Boarfish are widely distributed, spanning from Norway to Senegal in the Northeast Atlantic, including the Mediterranean and Aegean Seas, as well as around the Azores, Canaries, Madeira, and the Great Meteor Seamount (Holgersen 1954; Quéro 1986; Kaya and Özaydin 1996). Boarfish inhabit shelf seas and slopes and can be found from 40 – 600 m over rock, coral and sand (Blanchard and Vandermeirsch 2005; O'Donnell *et al.* 2012).

Although sporadically recorded in fisheries around the British Isles as far back as the 1800s, with occasional temporary population expansions, it wasn't until the 1980s and 1990s that the boarfish population of the Northeast Atlantic grew significantly. In the Bay of Biscay Blanchard and Vandermeirsch (2005) found that the boarfish abundance increased from 7 to 1 500 individuals per haul between the early 1970s and 2000. This change has been attributed to increasing sea water temperatures coinciding with low predation and targeted fisheries exploitation (Blanchard and Vandermeirsch 2005). Estimates from Spanish discard data suggest that their numbers peaked in the late 1990s and have since decreased slightly (Valeiras *et al.* 2012). However, significant numbers are still regularly recorded. In 2016, the Western European Shelf Pelagic Acoustic Survey carried out 47 trawl hauls, of which 15 contained >50% boarfish. From this, total stock abundance was estimated at 1 157 000 individuals (O'Donnell *et al.* 2016).

Boarfish are zooplanktivorous. Their diet consists primarily of copepods, notably *Calanus helgolandicus*, mysid shrimps, euphausiids, and hyperiid amphipods (Santos and Borges 2001; Fock *et al.* 2002; Lopes *et al.* 2006).

With their well-armoured bodies, boarfish seem an unlikely target for predation. However, a significant number of larger fish species as well as birds have been recorded predators. These recordings have primarily been taken around the Azores, where the boarfish have been recorded as an important prey item for species such as tope shark (*Galeorhinus galeus*), thorn-back ray (*Raja clavata*), conger eel (*Conger conger*), bigeye tuna (*Thunnus obesus*), and blackspot seabream (*Pagellus bogaraveo*) (Clarke *et al.* 1995; Morato *et al.* 1999; Morato *et al.* 2001; Morato *et al.* 2003; Arrizabalaga *et al.* 2008) as well as Cory's shearwaters (*Calonectris diomedea*) and more surprisingly the common tern (*Sterna hirundo*) (Granadeiro *et al.* 1998; Granadeiro *et al.* 2002; Ceia *et al.* 2015). It is suggested that boarfish may be prolific in predator diets in this region due to a lack of other available food sources (Nolan

2014). Off the coast of Portugal recorded predators include hake (*Merluccius* L.), mackerel (*Scomber scombrus* L.), conger eel (*Conger* L.), monkfish (*Lophius piscatorius* L. and *Lophius budegassa* Spinola) and catshark (*Galeus melastomus* Rafinesque), as well as yellow-legged gulls (*Larus michahellis*) (Santos and Borges 2001; Cabral and Murta 2002; Alonso *et al.* 2015). No known studies in Irish waters of the Northeast Atlantic have highlighted boarfish as an important prey species. In fact, studies investigating the diets of conger eel, tope shark, thornback ray and hake in this region have not found any boarfish specimens (Nolan 2014). However, this lack of evidence should not lead directly to the assumption that boarfish are not a component of predatory fish diets in this area.

2.4 Body composition

Boarfish are, on average, ca. 10.0 cm long and weigh 50 g. Their muscle content is lower than yields from most other commercialized fish (40 – 60%), averaging at 22.5% (Blanco *et al.* 2015). *C. aper* are characterised by robust dorsal and pectoral spines. They also have high bone content, with 6% bone found in the edible bone-in parts (Cunningham and Gormley 2015a). Protein content has been calculated between 17.6 to 18.7%. Oil content appears variable, with reports as low as 4.1% and up to 9.4%. Within the lipid profile there are 0.32% saturated fats, 0.18% omega-6 poly-unsaturated fatty acids (PUFA) and 0.48% omega-3 PUFA. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two omega-3 PUFA of special interest because of the roles they play in human health and nutrition. Looking specifically at these, their content in boarfish has been reported at between 42 – 143 mg 100 g^{-1} and 192 – 439 mg 100 g^{-1}, respectively. The values above were reported by the Irish semi-state seafood organisation Bord Iascaigh Mhara (BIM) and a single study carried out by University College Dublin, that was published in a magazine article in 2015 (BIM 2015; Cunningham and Gormley 2015a). To the best of our knowledge, there have been no peer-reviewed papers published to date that report the proximate composition of *C. aper*.

Although a number of studies have investigated the diets (Santos and Borges 2001; Fock *et al.* 2002; Lopes *et al.* 2006, and those referenced within) and intestinal parasites (Nicoll 1914; Baylis and Jones 1933; Trilles 1964; Bray and Gibson 1997) of boarfish, none to-date, that we have been able to find, describe the digestive anatomy or the associated gut microbiota of this species.

3. BOARFISH, THE FISHERY

Historically, considered rare in the Northeast Atlantic, boarfish were caught sporadically in large numbers in the second half of the 19th century and by the middle of the 20th century there were reports of increased abundance (Coad *et al.* 2014). Aside from a targeted Portuguese fishery in the 1960s, boarfish were largely caught and discarded as nuisance by-catch in pelagic and demersal fisheries (Blanchard and Vandermeirsch 2005). However, an opportunity was recognised when consistent increases in abundance were noted (Coad *et al.* 2014).

Irish vessels began exploratory boarfish fishing trips in the 1980s, and the Dutch began investigations in the mid-1990s into the development of a market for human consumption (O'Donnell *et al.* 2012). In 2001 the first targeted fishery began (Nolan 2014). Landings remained low (100 – 700 tonnes (t) per year) until after 2006, when they began to increase exponentially reaching a high of 207 882 t landed in 2012 (ICES 2012; ICES 2016a; Figure 2.1.3.).

Before 2011, there was no management of the Northeast Atlantic boarfish fishery and little scientific knowledge of the species on which to base any regulations. Scientists and fishers in Ireland and Denmark have collaborated and collected data on the boarfish stock since 2005 (Stange 2016). Originally, the boarfish stock expansion was explained by warming sea temperatures (Blanchard and Vandermeirsch 2005), but it is now thought that the causal mechanisms are more complex, and continued stock surveys and research are urgently needed to better understand the life history and spawning biology of this species. In the meantime, the European Council took a precautionary measure in 2011 and reduced the total allowable catch (TAC) to 33 000 t (Stange 2016). In 2012, the Pelagic Advisory Council, with assistance from Irish and Danish scientists, presented a long-term management plan for the fishery. This provided a decision framework adaptable for varying levels of data availability. The plan was forwarded to the International Council for Exploration of the Sea (ICES) who will review its validity before using it to advise on stock management.

Ireland, Denmark, and Scotland are the principle countries involved in the targeted boarfish fishery, and Ireland receives the majority of the quota. The total EU quota for Northeast Atlantic boarfish in 2014 ended at 127 509 t, with Ireland landing 88 115 t. In 2015,

the TAC was set at 53 296 t. Also, in 2015, however, the EU landings obligation, a part of the new Common Fisheries Policy (Regulation (EU) No 1380/2013), required all pelagic quota species caught during pelagic operations targeting pelagic quota species to be landed. Therefore, The Netherlands (375 t), England (104 t) and Germany (4 t) reported boarfish landings for the first time (ICES 2016b). Ireland still received over half of the quota; 36 830 t. This was adjusted throughout the year, and Ireland's final landing quota for 2015 was 45 640.5 t. However, interestingly, only 16 281 t were landed (SFPA 2015). The reasons behind this are believed to be two-fold. Fishers reported lesser availability of fishable aggregations, but the quota was also allocated to individual boats, and boats without the specialised fishing gear were unable to use their portion (ICES 2016b). In late 2016, the issue with quota was resolved by removing individual boat allocation.

As a new fishery, there are multiple components that are unknown, and management will need to continue making adjustments as required. In 2016, ICES recommended a TAC of 42 636 t. Thus far, quotas as well as landings have oscillated. Management plans, including closed seasons, have been implemented, new regulations have altered the participants in the fishery on a national and international scale, and data collection has incrementally increased, with plans to include this stock as one to be sampled under the data collection framework (DCMAP) from 2017 (ICES 2016b). The continuation and ultimate success of boarfish as a targeted fishery, however, will reside on reliable landings and its overall profitability. Currently, most of the catch is still landed in Denmark to be used as fishmeal for the aquaculture industry (Stange 2016).

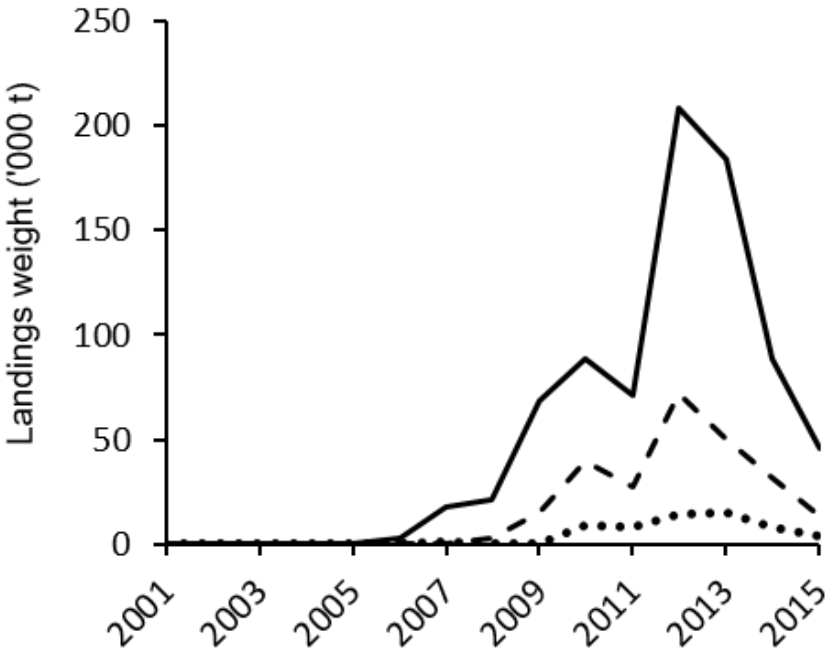

Figure 2.1.3. Boarfish landings by vessels registered in Ireland (——), Denmark (– – –), and Scotland (- - - - -) from the Northeast Atlantic between 2001 and 2015.

4. VALORISATION POTENTIAL

As is the case for many nations, Ireland's annual TACs and quota allocations for demersal and pelagic species have been decreasing over the past decade in response to growing concerns about the over-exploitation of numerous fish stocks (STECF 2014). Although the pelagic species dominate Irish landings in terms of weight, it is only mackerel and horse mackerel (*Trachurus trachurus*), along with Norway lobster (*Nephrops norvegicus*), monkfish, and hake that contribute considerably to the value of landings (Gerritsen and Lordan 2014). The only Irish quota for a pelagic species that increased between 2014 and 2015 was for blue whiting (*Micromesistius poutassou*; from 21 934 t to 23 313 t; DAFM 2014a; Table 2.1.1.). This suggests that if Ireland wants to increase the profitability of its fisheries sector while complying with scientific advice of minimum sustainable yield, they will need to look at innovative and sustainable ways of increasing the value of their landings, especially in the pelagic fisheries.

Since boarfish became a targeted fishery in the Northeast Atlantic, the catch has primarily been landed in Denmark to be processed for fishmeal (White *et al.* 2010). However, concurrently with other low-value fish that were previously discarded, there is growing interest in investigating improved valorisation of this fishery. A number of options exist; however, to guide appropriate investment an understanding of the composition of boarfish must first be acquired.

Aside from the cursory investigations into the proximate composition of boarfish, discussed above, a number of recently published studies have focussed on extracting and analysing boarfish protein (Hayes and Flower 2013; Blanco *et al.* 2015; Ojha *et al.* 2016). Non-hydrolysed boarfish protein meets the FAO's 35% recommendation for essential amino acid content (Hayes and McKeon 2014). Hayes and Flower (2013) provided the amino acid content of boarfish myofibrillar protein extract. The only amino acids that are not recorded are tryptophan and glutamine. Similarly, Blanco *et al.* (2015) and Ojha *et al.* (2016) analysed the total and free amino acid content after hydrolysing boarfish protein and observed similar trends as Hayes and Flower (2013) (Table 2.1.2.).

The heavy metals content of boarfish has also been reported. These fish were found to contain 0.01 - 0.02, < 0.12, and < 0.05 - 0.08 mg kg^{-1} of cadmium, lead and mercury, respectively (Cunningham and Gormley 2015b). Importantly, these levels are within safe

limits for consumption when compared to the maximum levels (mg kg^{-1} wet weight) for fish according to the Food Safety Authority Ireland (0.05, 0.3 and 0.5, respectively; FSAI 2009).

There are a great number of potential products and by-products that could be produced from boarfish (Figure 2.1.4.). Currently there are two principle lines of investigation: (i) developing a product and market for direct human consumption and (ii) bioprocessing the raw material to generate compounds that can be used in the food, feed, and pharmaceutical industries. Studies to date have conflicting views on the ideal route for valorisation. One assessment of discarded species of Spanish and Portuguese *métiers* suggested that boarfish would be optimally valorised as a direct human consumption product or alternatively be processed for use in products such as fishmeal or surimi, with little or no interest of valorisation as bio-compounds (Ordóñez-Del Pazo *et al.* 2014). While another study, taking into account costs of production, promoted high added-value bio-compounds (Antelo *et al.* 2015).

Table 2.1.1. EU TACs and quotas for the Irish fishing fleet's principle pelagic stocks in 2014 and 2015.

		Whiting	Mackerel	Horse mackerel	Blue whiting	Herring	Boarfish
2015 (19th Oct)	Ireland	6,507	90,153	25,779	25,202	22,115	45,641
2015	Ireland	5,154	89,220	21,968	23,313	19,433	36,830
	EU Total	35,988	NA	174,004	NA	774 932	53,296
2014	Ireland	5,880	104,967	30 551	21,934	30,690	88,115
	EU Total	41,357	NA	202,140	1,200,000	1,203,576	127,509

Table 2.1.2. Total amino acid profile (mg g^{-1}) of protein hydrolysates obtained from whole boarfish (Ojha *et al.* 2016).

Amino Acids	mg/g
Asparagine	0.349
Threonine	0.311
Serine	0.474
Glutamic	1.240
Glycine	0.342
Alanine	0.512
Cysteine	1.438
Valine	0.639
Methionine	1.675
Isoleucine	2.240
Leucine	1.112
Tyrosine	1.951
Phenylalanine	1.930
Histidine	1.977
Lysine	0.537
Arginine	0.481
Proline	0

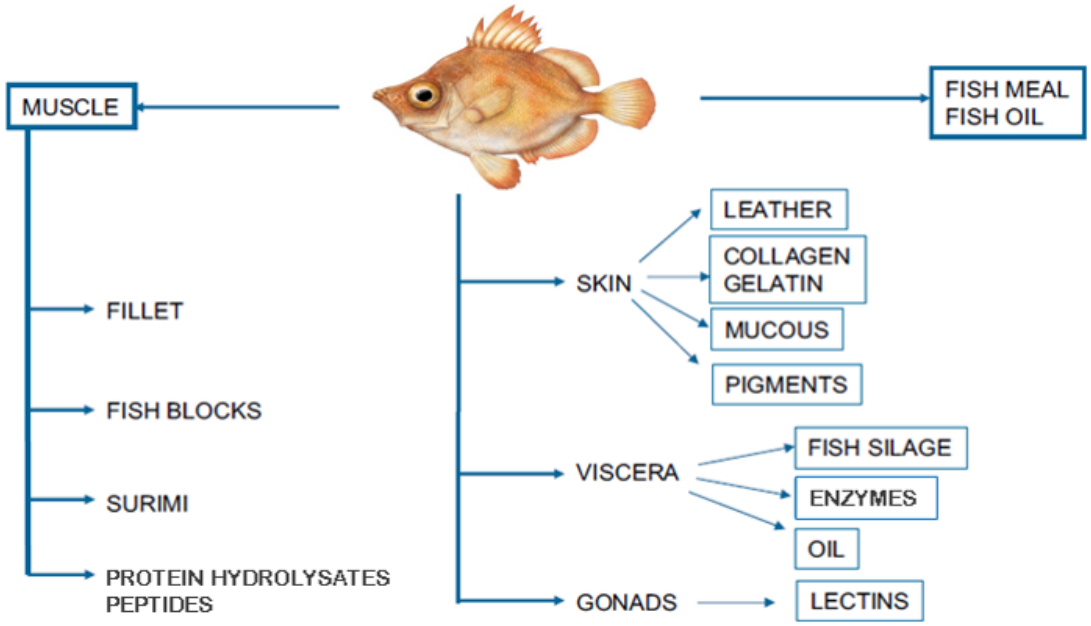

Figure 2.1.4. Scheme of potential products and by-products of boarfish (*Capros aper*); adapted from Blanco *et al.* (2007).

4.1. Human consumption

4.1.1. Whole fish & mince blocks

Research on the organoleptic and nutritional properties along with food applications of boarfish is currently being performed in Spain and Ireland (Ordóñez-Del Pazo *et al.* 2014; Cunningham and Gormley 2015a; Cunningham and Gormley 2015b). Simultaneously, potential markets within Europe, Asia, and Africa have been investigated (DAFM 2014b; Ordóñez-Del Pazo *et al.* 2014; Saumell 2014). BIM in Ireland has driven the production of direct human consumption products and has made efforts to build a strong market interest for them in China. They promoted two processed products: (i) a mince block which can be further prepared in a variety of ways (e.g. into breaded consumables, fish cakes, surimi or dried products) and (ii) headed and gutted fish which can be pan-fried or breaded (BIM 2015). They have worked with Chinese celebrity chefs to develop recipes specifically for the Asian market and have engaged with TMALL® (www.tmall.com), the largest Chinese language website for business-to-consumer online retail, to build potential sales through this channel (DAFM 2014b). Boarfish have received high scores in sensory studies, and very positive customer feedback has been received in the Chinese market (DAFM 2014b; Cunningham and Gormley 2015b).

4.1.2. Surimi, sauce & flavourings

Other potential products for human consumption include surimi, fish sauce and flavourings. Surimi is a traditional Japanese dish made from ground fish fillets that are rinsed and flavoured before being made into cakes. Surimi-based products have increased significantly in popularity in Europe, along with fish sauces and flavourings (Karadeniz and Kim 2014). At this point, there is no known production of these products from boarfish and an analysis of economic and environmental costs of processing boarfish found that surimi production would be more environmentally costly in comparison to producing bioactive peptides from the fish muscle (Antelo *et al.* 2015). This is due to the large volumes of water required for the production of surimi (Santana *et al.* 2012).

4.2. Bioprocessing

4.2.1. Functional & bioactive peptides

The production of protein hydrolysates, peptides and even free amino acids from boarfish has been the topic of some recent papers (Hayes and Flower 2013; Blanco *et al.* 2015; Ojha *et al.* 2016; Parthsarath *et al.* 2016). Interest lies in end-products that have functional properties that would be beneficial as a functional food ingredient or bioactive properties that could be purified and concentrated for medicinal uses. Boarfish protein hydrolysates extracted with Alcalase and bovine trypsin, as well as a crude pancreas extract enzyme have been tested for solubility, foaming capacity, emulsion capacity, and antioxidant activity. Enzyme choice affected the amount of protein obtained, the degree of hydrolysis, as well as the functional properties. The antioxidant activities, however, were similar for all enzymes (Blanco *et al.* 2015). Protein hydrolysates and related filtrates produced using the enzymes Alcalase CLEA, Papain and Protease AP, were found to have angiotensin-converting-enzyme (ACE) inhibitory activities comparable to those shown previously by kidney bean and pea protein hydrolysates (Hayes and Fagan 2014). Analysing the optimum route for processing boarfish in a bio-refinery, Antelo *et al.* (2015) reported that a higher economic profit would be attained from valorising specific fish fractions (i.e. the muscle), and congruently the optimal environmental solution was one that processed at least 80% of the biomass to obtain bio-peptides and left the remaining biomass unprocessed.

4.2.2. Sports nutrition

Protein hydrolysates are a very accessible source of nutrition. They are used in the food industry as a functional ingredient and a fortifying supplement (Ghaly *et al.* 2013). However, they can have a characteristic bitterness which, to a degree, inhibits their use in that area (Kristinsson and Rasco 2000). Fish protein hydrolysates (FPH) are most commonly used as an additive in animal feeds; however, recent processing developments that allow the deodorizing of fishy smells from the FPH have unlocked opportunities for their application in human food applications (Karadeniz and Kim 2014). Sports nutrition is a fast-growing market, and marine proteins can be considered an ideal source of raw material. To date, few marine-derived sports nutrition supplements have reached the market. However, marine animals have higher protein content than most terrestrial animal materials. Furthermore, their

proteins are highly digestible, and they have excellent essential amino acid profiles that closely approximate human dietary requirements as set by the World Health Organisation (WHO) (WHO *et al.* 1985; Sultana *et al.* 2014). Boarfish muscle protein contains ample levels of leucine, isoleucine and valine (Table 2.1.2.). These branched-chain amino acids are desirable in sports protein powders as they make up one-third of skeletal muscle protein, and an increasing amount of literature suggests that leucine plays a critical role in stimulating protein synthesis (Campbell *et al.* 2007).

4.2.3. Growth media

The nutritional attributes of boarfish protein hydrolysates could be exploited in the production of growth media. Growth media can be either completely natural or synthetic with the addition of some natural products (Arora 2013) but must include all the nutrients and molecules required for cell survival, including amino acids, glucose, salts and vitamins. Peptones are an important component of growth media. Historically, these peptones have largely come from bovine and porcine origins. With increasing demands for higher volumes of low-cost media and issues such as generating halal and kosher products as well as concerns over diseases such as bovine spongiform encephalopathy (BSE), alternative peptone sources are gaining greater attention (Safari *et al.* 2012). In addition, due to prolific viral contamination, biopharmaceutical producers have largely moved away from animal-based serums in favour of media that is protein and animal-free (Wessman and Levings 1998). However, serum or protein-free media are unable to sustain cultivation of most animal cell lines without the addition of peptones (Siemensma *et al.* 2008). Protein hydrolysates not only provide amino-acids and peptides but also carbohydrates, vitamins, minerals, and a range of undefined components (Siemensma *et al.* 2008). It should be noted, however, that protein hydrolysates have limitations in industrial use. Their current mechanism of production causes batch variability that can lead to product inconsistencies (Grosvenor 2008). However, some companies are managing to overcome these challenges with refined processes and optimized enzymatic hydrolysis (Grosvenor 2008; Siemensma *et al.* 2008).

4.2.4. Collagen & gelatine

Other compounds that could be attained from boarfish include collagen and gelatine, enzymes and oil. Currently, there is limited data on the extraction of collagen and its degradation product gelatine from boarfish; however, it may become a principle area of interest. Due to their gelling and stabilising properties, collagen and gelatine have many uses in the food, cosmetic, and pharmaceutical industries (Blanco *et al.* 2007; Ferraro *et al.* 2010; Hayes and McKeon 2014). They were also found to be a good source of ACE inhibitors and can be used as nutritional supplements to improve nail and hair growth (Kim and Mendis 2006). Although currently fish-sourced collagen and gelatine only account for about 1% of the global market, the spread of diseases such as BSE and a growing population with kosher and halal requirements means that there is considerable room for growth in the area (Kim and Mendis 2006; Blanco *et al.* 2007).

4.2.5. Enzymes

Proteases are enzymes that break peptide bonds. Within industry, they are the most important type of enzymes, accounting for about 50% of the total industrial enzyme market (Turk 2006). They are derived from microbial, plant, or animal sources. Digestive proteolytic enzymes derived from fish viscera include pepsin, trypsin, chymotrypsin, gastricins and elastase (Blanco *et al.* 2007). Trypsins are highly sought after because they have a high activity over a wide range of pH and temperature conditions and exhibit high catalytic activity at relatively low concentrations (Bougatef 2013). These enzymes are used for applications in the detergent, food, agro-chemical, and pharmaceutical industries (Ktari *et al.* 2012; Ferraro *et al.* 2013). There are major opportunities for developing new niche markets around marine-derived enzymes (Ferraro *et al.* 2010) and boarfish-derived proteases may be a viable commodity that could be exploited in the future.

4.2.6. Fish oil

Fish oils are a popular bio-product used in a variety of markets since they have industrial, food, feed and aquaculture, and nutraceutical applications (Kim and Venkatesan 2014). Fish oil is an excellent source of the omega-3 PUFA, EPA and DHA (Kim and Mendis 2006) and boarfish is no exception (BIM 2015; Cunningham and Gormley 2015a). Oil could be extracted

from whole boarfish or specifically from the skin or liver. Boarfish contain, on average, 9% fat (BIM 2015). In comparison, Atlantic salmon fat content ranges from 6 to 10.5% (Exler and Pehrsson 2007). Boarfish are an ideal fat source for human and animal nutrition and currently boarfish landings are directed into salmon aquaculture feeds (Ordóñez-Del Pazo *et al.* 2014). Value could be added in this line by producing high-value speciality feeds to be used at critical points of production, e.g. finisher feeds which have been shown to be effective in boosting animals' health and condition and, therefore, producing high quality end-products (Codabaccus *et al.* 2013; Karadeniz and Kim 2014).

Alternatively, products for human nutrition could be produced. The benefits of fish oil supplementation in the diet are supported by a plethora of papers published since the first studies by Burr and Burr (1929). It is widely accepted within the general public today that increasing consumption of omega-3 oils can help prevent inflammatory and cardiovascular diseases. The main challenges related to such a valorisation route is in developing methods of extraction, purification and encapsulation that will consistently produce high quality oil (Kim and Mendis 2006). Soerensen and Jensen (2014) have patented a process for the isolation of a phospholipid and for producing a PUFA-enriched fraction from fish oil that may be derived from boarfish. New innovative processing methods such as supercritical fluid extraction and micro-encapsulation are advancing improvements in this area continually.

5. CONCLUDING REMARKS

The Northeast Atlantic boarfish fishery is an interesting case study for the development of a new large-scale fishery within an established fishing industry. It is an excellent example of fishers creating new opportunities in an increasingly restricted sector. This initially burgeoning fishery has invariably met with some challenges. Abundance has proved variable, with insufficient numbers to reach TACs on some years, and there have been issues with potential bycatch of other TAC species, largely mackerel and herring (*Clupea harengus*). Furthermore, the armoured bodies of these small fish mean that specialised equipment is required at both the fishing and processing stages. This necessary investment of assets, inevitably, limits those capable of participating in the fishery.

Increased effort has been allocated to collecting information on this species. In 2011, fishery-independent acoustic surveys were initiated, some discard data have been available since 2015, and in 2017, boarfish were added to the EU's data collection framework (DCMAP; ICES 2016b). However, surveys and data are only being included from ICES Subareas VI – VIII, even though stock is believed to cover a greater area, e.g. into Subarea IX. Since data are still limited, a precautionary model is being used, along with a static age – length key, to guide quota-setting advice. It is hoped that the continual collection of data will allow for a more tailored management plan in the near future and will help develop an understanding of the population dynamics that will provide confidence for investors.

Evidence to date suggests that there is high potential for adding value to this fishery. Increasing interest in this species has stimulated investigations into a number of valorisation routes. The early successes presented here derive from cross-sector collaboration. Irish and other EU governments have supported the growth of this fishery and helped to find and build new markets for it. Scientists have been building a knowledge base of the species as well as investigating its valorisation potential. They have also been working with fishers and regulation authorities to establish appropriate quotas and create management plans that will ensure the fishery’s sustainability.

Product and market developments derived from boarfish are transferrable to many pelagic fisheries around the world. These open up huge opportunities for increasing profitability within a sector that will continue to experience constraints on raw material supply.

Acknowledgements

We would like to gratefully acknowledge the funding of this work by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. via the IRC Enterprise Partnership Scheme.

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Chapter 2.2

The gut microbiota of marine fish

Published: *Frontiers in Microbiology* (2018), **9**.
doi: 10.3389/fmicb.2018.00873

ABSTRACT

The body of work relating to the gut microbiota of fish is dwarfed by that on humans and mammals. However; it is a field that has had historical interest and has grown significantly along with the expansion of the aquaculture industry and developments in microbiome research. Research is now moving quickly in this field. Much recent focus has been on nutritional manipulation and modification of the gut microbiota to meet the needs of fish farming, while trying to maintain host health and welfare. However, the diversity amongst fish means that baseline data from wild fish and a clear understanding of the role that specific gut microbiota play is still lacking. We review here the factors shaping marine fish gut microbiota and highlight gaps in the research.

1. A HISTORICAL OVERVIEW

Fish and other marine animals have a unique and intimate interaction with their surrounding environment and, in turn, with the microorganisms that co-exist there. The world's oceans are teeming with microorganisms. It is estimated that 3.6 x 10^{30} microbial cells account for more than 90% of the total oceanic biomass, while the number of viral particles may be one hundred-fold greater (ICES 2011). The relationship that fish have with surrounding microorganisms can be mutualistic or pathogenic. Like humans and other mammals, fishes associated symbiotic gut microbiota play a role in nutritional provisioning, metabolic homeostasis and immune defence (Gómez and Balcazar 2008; Sullam *et al.* 2012).

Fish originated over 600 million years ago and include nearly half of all extant vertebrates (Nelson 2006; Sullam *et al.* 2012). Over three billion people around the world depend on fish for at least 20% of their protein intake and approximately 20 kg of fish is consumed per capita per annum (FAO 2016). Wild-caught fisheries can no longer support the world's seafood consumption thus, unsurprisingly, aquaculture is reported to have contributed 43.1% of global fish production in 2013 (FAO 2015). The vast diversity that fish contribute to the sub-phylum chordata, our reliance on fish as a food source and the environmental changes that are being inflicted on them highlight the need to consider them in the growing field of host microbial research.

Research into the gut microbiota of fish dates back to the early half of the 20th century but more recently interest in this area has grown at a significant rate coinciding with the expansion of the aquaculture industry. Indeed, the first works on this topic were published in the late 1920s and 1930s (Reed and Spence 1929; Gibbons 1933) and investigated the intestinal and "slime flora" of fish. There were some further exploratory studies during the 1950s and 60s; Margolis (1953) investigated the effect of fasting on the intestinal flora, Colwell (1962) examined the intestinal flora of Puget Sound fish and Simidu and Hasuo (1968) examined the salt dependency of fish flora. In the following decade, the studies became more applied, with interest in how the gut microbiota changed with diet (Sera and Ishida 1972), how the microbiota changed in farmed fish (Gilmour *et al.* 1976) and how animals succumbed to infection (Boulanger *et al.* 1977; Olivier *et al.* 1981).

In the early 1990s the first reviews on this topic were published (Cahill 1990; Ringø *et al.* 1995). They provided a comprehensive overview of the studies to date; however, they

consequentially reported that bacterial levels in the gut of fish were low and appeared to be derived from the surrounding environment or diet (Cahill 1990; Ringø *et al.* 1995). These conclusions were made based on research using culture-dependent methods, but we now know that no more than 10% of microorganisms could be isolated and cultured under such laboratory conditions as were used then (Amann *et al.* 1995). Analytical techniques have evolved significantly since then and it is now reported that cultivable microorganisms represent < 0.1% of the total microbial community in the gastrointestinal (GI) tract of some species of fish (Zhou *et al.* 2014). Despite this, many recent studies continue to report results obtained through culture-based approaches, inferring microbiota function from data derived from bacterial growth studies performed under artificial environmental conditions (Clements *et al.* 2014). Today a wide variety of culture-independent techniques are available for analysing fish microbiota. These have been discussed in detail in some recent reviews (Zhou *et al.* 2014; Tarnecki *et al.* 2017). Briefly, they include quantitative real-time PCR (qPCR), used for quantitative analysis of taxa; clone libraries for identification of microbiota composition; finger-printing methods such as temporal temperature gradient electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE), and fluorescent in situ hybridization (FISH) used to determine the abundance of particular taxa, total microbial levels and assess bacterial–host interactions at the mucosal brush border (Zhou *et al.* 2014; Wang *et al.* 2018). Next-generation sequencing is the latest method of molecular analysis. It is beginning to be used more frequently in studies on fish and Ghanbari *et al.* (2015) have discussed its potential in this field, including the opportunity for rapid and cost-effective acquisition of in-depth and accurate sequence data that provide greater information on even low abundance microbiota as well as the genetic and metabolic potential of the species present.

With the development of these new molecular techniques and the exponential growth of aquaculture, the research of fish gut microbiota has expanded dramatically over the previous decades. In this review, we focus on the gut microbiota of marine species. We have included anadromous salmonids in our discussions but do not focus on them or the novel changes that these fish experience in their gut microbiota as they develop and move across habitats. This is an area which has thus far been poorly understood but is receiving new interest in some recently published articles; Llewellyn *et al.* (2016), Dehler *et al.* (2017), and Rudi *et al.* (2018). Even when looking specifically at saltwater fish, the diversity is enormous. In this review, we discuss the trends and supporting findings in the current literature, but also

highlight the contradictory studies that are inevitable within such a diverse group. Overall, the purpose of this review is to provide an overview of the fish alimentary canal, the gut microbiota within it and how the diversity of these communities develops with life stage and is affected by factors including trophic level, season and captive-state. Finally, we review the latest research that investigates the dietary manipulation of gut microbiota in aquaculture species and discuss future perspectives.

2. THE FISH ALIMENTARY CANAL

There is no single blue print for the alimentary canal of a fish; fish biology varies greatly with differing life histories, ecology and environmental factors. Filter feeders, parasites and predators as well as herbivorous and carnivorous fish exist, and each has an appropriately adapted digestive system. Regardless of diet, the gut of some fish consists simply of a short tubular intestine, e.g., parrotfish (*Scarus radicans*) (Horn *et al.* 2006). However, the majority of fish alimentary canals are divided into topographical regions with unique roles. All fish alimentary canals begin with the buccal and pharyngeal cavities of the head-gut. From here, the gut can be loosely divided into the fore-, mid- and hind-gut which include various digestive organs that particular fish either possess or lack. The foregut, beginning at the posterior edge of the gills, often consists of the oesophagus, stomach and pylorus. However, it is estimated that 20% of fish species lack a true stomach (Wilson and Castro 2010). Species that have evolved such simple digestive tracts include fish in the *Gobiidae* and *Blennidae* families (Figure 2.2.1.). This lack of stomach in some species may be counteracted by other adaptations such as well-developed pharyngeal teeth, pharyngeal pockets, secretory glands in the oesophagus or a muscular gizzard (James 1988; Kapoor and Khawna 1993; Stevens and Hume 2004). When the stomach is present it is usually one of three shapes; straight, U-shaped, or Y-shaped with a gastric cecum (Figure 2.2.1.). Straight stomachs are relatively rare but can be found in some freshwater species as well as marine fish such as mullet (*Mugil*) anchovy (*Engraulis*) and menhaden (*Brevoortia*). The U-shaped stomach is more frequently seen and is common in omnivores and carnivores such as seabass (*Dicentrarchus*) and salmonids. The Y-shaped stomach is proposed to be an adaptation of macrophagous predatory fish for storage of large pieces of food and is found in eels (*Anguilla*) (Stevens and Hume 2004).

Generally, no definitive distinction exists between the mid- and hind-gut. However, the former is the longest portion of the gut, which includes the pyloric ceca when present. The mid-gut is where the majority of digestion occurs, and the pyloric ceca are thought to be organs acquired to produce a greater surface area for absorption. Although not always obvious, this section often ends with an increase in tube diameter, indicating the beginning of the hindgut (distal intestine and anus). Fish intestines vary dramatically in length. When longer than the visceral cavity, the intestines are coiled in a loop unique to each species. Gut length is loosely associated with diet and as a guide is three times longer than body length in

herbivorous fish, one to three times in omnivores and approximately equal in carnivores (Bone *et al.* 1995; Karachle and Stergiou 2010).

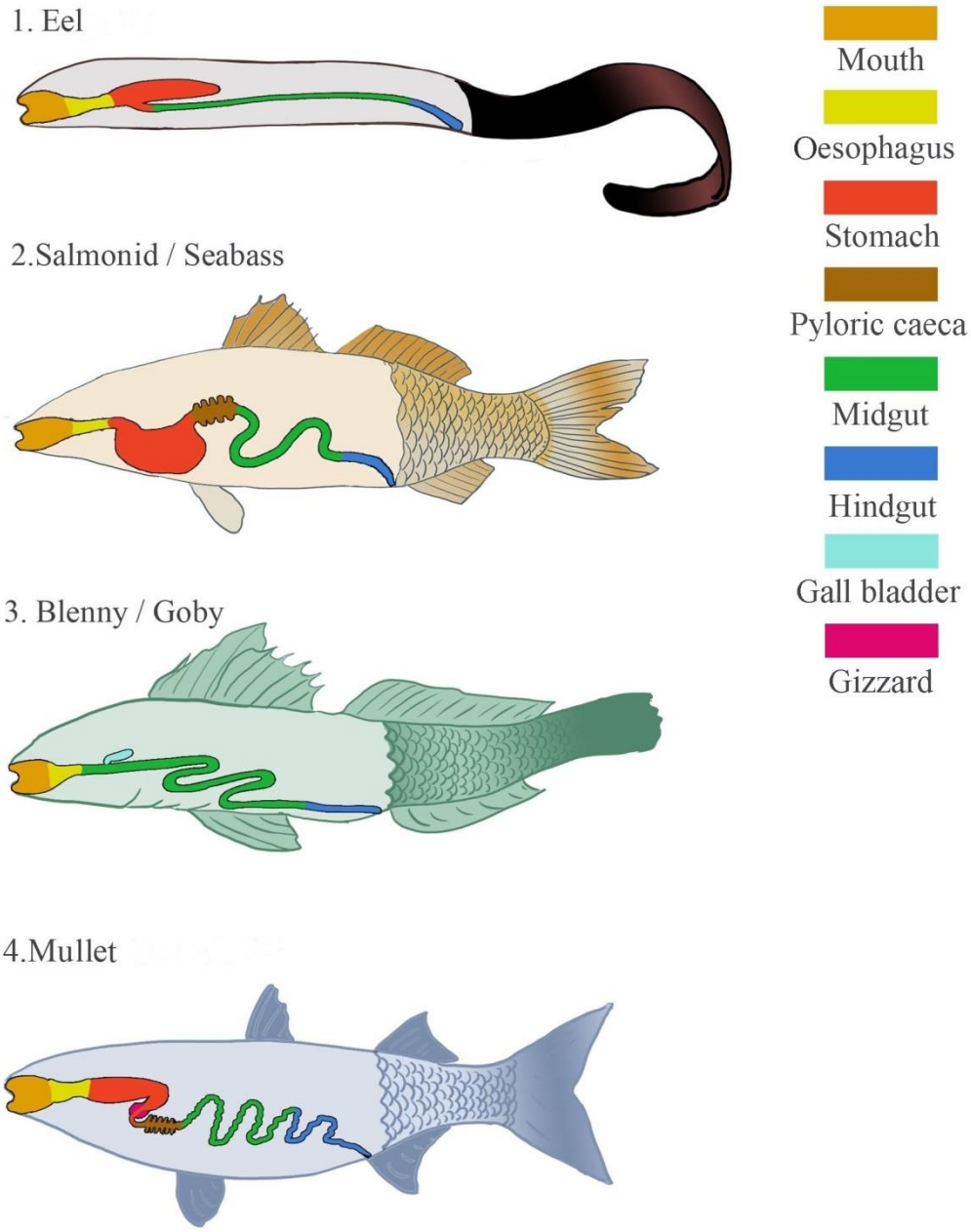

Figure 2.2.1. Diagrammatic representation of the different types of digestive systems that can be found in marine fish, including digestive organs that may or may not be present.

3. DEVELOPMENT OF THE GUT MICROBIOTA IN FISH

Microbial colonisation of fish larvae originates from the eggs, the surrounding water and the first feed. Some initial studies investigating bacteria associated with fish eggs suggested that the dominating species at this point included *Cytophaga, Flavobacterium*, and *Pseudomonas* (Bell *et al.* 1971; Austin 1982). While some recent studies provide correlating results (Kubilay *et al.* 2009), others differ completely (Romero and Navarrete 2006; McIntosh *et al.* 2008). Even some early studies recognised that inter-species variation existed. For example, Hansen and Olafsen (1989) observed differences in the bacterial colonisation of cod (*Gadus morhua L.*) and halibut (*Hippoglossus hippoglossus*) eggs. The initial colonising bacteria are now accepted as species-specific, with differences controlled by variation in binding glycoproteins on the egg surface (Larsen 2014). In addition, the microbiota of the surrounding water dictates what bacteria encounter the eggs and consequently have the opportunity to colonise. Upon hatching, sterile larvae take in the chorion-associated bacteria, which become the first colonisers of the developing gastrointestinal tract (GIT). Subsequent inhabiting bacteria are acquired when the fish larvae begin to drink water to control osmoregulation and the microbiota then becomes further diversified through feeding (Hansen and Olafsen 1999). To begin, the GIT of newly hatched larvae tends to contain few bacteria (Ringø *et al.* 1991). Numerous studies have shown that diet is influential in shaping the gut microbial community and from first feeding substantial diversification occurs (Blanch *et al.* 1997; Korsnes *et al.* 2006; Reid *et al.* 2009; Lauzon *et al.* 2010). Interestingly, like in humans (Yatsunenko *et al.* 2012), it appears the diversity of bacteria increases as fish develop. In Ringø and Birkbeck's (1999a) review of the Intestinal microflora of fish larvae and fry, they summarised 24 studies that reported the bacterial genera in the intestinal tract of freshwater and marine fish at the larval and fry stages. In the 11 marine species, the bacteria most frequently reported were *Vibrio* (16 times), *Pseudomonas* (9), *Aeromonas* (9), *Cytophaga* (8), *Flavobacterium* (7) and the family *Enterobacteriaceae* (7). On average, the studies reported three to four genera/families (Table 2.2.1.). A comparison of the gut microbiota of 12 (adult) bony fish found bacteria representing 17 phyla, with most species having between 7 and 15 phyla, a far higher average than in the review of egg and larvae microbiota. While the microbial community changes with life stage and habitat, a relatively stable gut microbiota is established within the first 50 days of life for many species (McIntosh *et al.* 2008; Larsen

2014). A decisive study with zebrafish (*Danio rerio*) demonstrated this, reporting that a core microbial community is supported through host system selective pressures regardless of environmental parameters (Roeselers *et al.* 2011).

Table 2.2.1. Bacterial species isolated from the intestinal tracts of marine fish species at larval and fry life stages.

Fish species	Bacterial genera	References
Atlantic cod, *Gadus morhua*	*Vibrio/Aeromonas*	Strøm & Olafsen (1990)
	Aeromonas, Pseudomonas, Cytophaga/Flexibacter, Lactobacillus	Strøm & Ringø (1993)
Atlantic halibut, *Hippoglossus*	*Cylophaga/Flexibacter/Flavobacterium, Vibrio/Aeromonas*	Bergh *et al.* (1994)
	Vibrio/Aeromonas	Bergh (1995)
Dover sole, *Solea*	*Pseudomonas/Alcaligenes, Vibrio/anaergenic Aeromonas, Moraxella, Enterobacteriaceae, Flavobacterium/Cytophaga,Moraxella, coryneforms*	Campbell & Buswell (1983)
Turbot, *Scophthalmus maximus*	*Vibrionaceae*	Nicolas *et al.* (1989)
	Vibrio alginolyticus, Aeromonas	Gatesoupe (1990)
	Vibrio pelagius	Blanch *et al.* (1991)
	Vibrio alginolyticus, V. natrigenes, V. anguillarum, V. fluvilis, V. pelagius, Aeromonas caviae, Acinetobacter	Munro *et al.* (1993)
	Vibrio alginotyticus, V. anguillarum, V. campelii, V. fluvilis, V. furnissi, V. harveyii, V. natrigenes, V. nereis, V. ordali, V. pelagius, V. splendidus, Vibrio, Aeromonas, Pseudomonas/Alcaligenes, Flavobacterium/Cytophaga, Enterobacteriaceae, Acinetobacter, Photobacterium, Moraxella	Munro *et al.* (1994)
	Aeromonas, Vibrio, Enterobacteriaceae, Cytophaga, Micrococcus, Staphylococcus, coryneforms	Ringø *et al.* (1996)
	Oxidative Gram-negative rods, V. natriegenes, V. eplagius, V. sophalmis, V. splendidus, V. mediterranei, V. anguillarum, V. alginolyticus	Blanch *et al.* (1997)
	Acinetobacter, Moraxella, Vibrio	Gatesoupe *et al.* (1997)
Herring, *Clupea harengus*	*Pseudomonas/Alteromonas, Flavobacterium*	Hansen *et al.* (1992)
Rockfish, *Sebastes schlegeli*	*Vibrio, V. anguillarum, V. alginolyticus, Pseudomonas, Acinetobacter, Flavobacterium/Cytophaga*	Tanasomwang & Muroga (1989)
Red seabream, *Pagrus major*	*Aeromonas, Vibrio, Pseudomonas, Enterobacteriaceae, Cytophaga*	Muroga *et al.* (1987)
Black seabream, *Acanthopagrus schlegeli*	*Aeromonas, Vibrio, Pseudomonas, Enterobacteriaceae, Cytophaga*	Muroga *et al.* (1987)
Milkfish, *Chanos*	*Pseudomonas, Vibrio, Enterobacteriaceae*	Fernandez *et al.* (1996)
Seabass, *Dicentrarchus labrax*	*Vibrio, Acinetobacter, Moraxella, Enterobacteriaceae*	Gatesoupe *et al.* (1997)
Wolffish, *Anarhichas lupus*	*Carnobacterium divergens*	Ringø & Johnsen (unpub. data)

Taken from Ringø and Birkbeck, 1999a.

4. STRUCTURE OF THE FISH GUT MICROBIOTA

The fish microbiome can be diverse, including protoctista, fungi, yeasts, viruses, and members of the Bacteria and Archaea (Merrifield and Rodiles 2015). Bacteria are the dominant microbiota of the fish intestine however (Rombout *et al.* 2011), and have been almost the sole focus of research in this field thus far.

Recent research has shown that fish hindgut microbial communities closely resemble those of mammals much more so than their surrounding environmental microbial communities (Ktari *et al.* 2012). Despite this, in mammals the dominant gut microbiota are anaerobes from the phyla Bacteroidetes and Firmicutes (Lozupone *et al.* 2012) whereas Proteobacteria are the prominent microbial phyla found in the fish GIT (Rombout *et al.* 2011). Proteobacteria, in addition to Bacteroidetes and Firmicutes, comprise 90% of the fish intestinal microbiota of the different species studied thus far (Ghanbari *et al.* 2015).

The density, composition and function of the microbiota change in the different sections of the fish GIT (Clements *et al.* 2014). Furthermore, there is a distinction between the allochthonous and autochthonous communities (Nayak 2010; Banerjee and Ray 2017). Allochthonous are the free-living, transient microbiota associated with the digesta, whereas, autochthonous microbiota colonise the mucosal surface of the digestive tract and make up the core community. The density of viable aerobic and anaerobic bacteria usually range from 10^4–10^9 colony forming units (CFU) g^{-1} of intestinal content, respectively (Skrodenytė-Arbačiauskienė 2007). This is notably lower than that of warm-blooded animals which are generally orders of magnitude higher (Nayak 2010). Similar to higher vertebrates, the densest population of microbes in teleost fish is located in the GIT. Previous studies have found increasing population sizes running distally along the GIT. Aerobic heterotrophs in the GIT of yellowtail (*Seriola* sp.) increased from 2 x 10^4 bacteria g^{-1} in the pyloric caeca and 2.5 x 10^5 bacteria g^{-1} in the stomach, finally, to 6.5 x 10^4 to 5.9 x 10^6 bacteria g^{-1} in the intestine (Sakata *et al.* 1978). This trend was also observed in herring larvae (*Clupea harengus*) (Hansen *et al.* 1992) and juvenile Dover sole (*Solea solea*) though not adults (MacDonald *et al.* 1986). The results of an analysis of the occurrence and distribution of enzyme-producing bacteria in the proximal, middle, and distal segments of the GIT of four brackish water teleosts (*Scatophagus argus, Terapon jarbua, Mystus gulio*, and *Etroplus suratensis*) showed that the density generally increased along the GIT (Das *et al.* 2014). Other studies also found similar trends

(Fidopiastis *et al.* 2006; Ringø *et al.* 2006; Bakke-McKellep *et al.* 2007; Hovda *et al.* 2007; de Paula Silva, Nicoli *et al.* 2011). Zhou *et al.* (2007) used 16S rDNA PCR-DGGE fingerprinting to study the autochthonous bacteria of *Lutjanus sebae* and *Ephippus sebae*. In this study, they found that the average number of different bacteria detected in each section increased along the digestive tract. In contrast, the opposite was found by Zhou *et al.* (2009) in yellow grouper (*Mycteroperca venenosa*) and no obvious trend was observed in juvenile Atlantic salmon (*Salmo salar*) (Navarrete *et al.* 2009). Numerous factors may have caused the deviating results of these studies; diet, which may be a significant one, will be discussed later in this review.

The community composition between sections of the fish GIT can also vary (Llewellyn *et al.* 2014). It has been suggested that the autochthonous microbiota can differ in particular, considering the variation in physiological environments between the different parts of the digestive tract (Clements *et al.* 2014). The stomach is often omitted from gut microbial composition analyses. However, a number of studies have included it in the past using culture-dependent techniques (Sera 1972; Sera and Ishida 1972; Austin and Al-Zahrani 1988; Ringø 1993; Ringø *et al.* 1998; Zhou *et al.* 2008; Zhou *et al.* 2009). There are also some more recent studies using culture-independent techniques to compare the microbial community in different gut segments, including the stomach. The dominant phyla in the stomach of gilthead seabream (*Sparus aurata*) were reported as Firmicutes, Proteobacteria and Bacteroidetes (de Paula Silva *et al.* 2011). However, a later study reported the dominant phyla to be Firmicutes, Proteobacteria, and Actinobacteria (Estruch *et al.* 2015). Both studies found *Vibrionaceae* to be a dominant family, reporting the genus *Photobacterium*. Aside from this, Estruch *et al.* (2015) also reported the family *Enterobacteriaceae*, the genera *Streptococcus* and *Clostridium* of Firmicutes and the genus *Corynebacterium* of Actinobacteria, whereas de Paula Silva *et al.* (2011) found bacteria relating to the genus *Vibrio* along with species from the family *Bacillales* of Firmicutes and the genus *Flavobacteriaceae* of Bacteroidetes. Results on stomach microbiota should be treated with caution. These two studies used stomach contents for analysis which is likely to be influenced by transient food. Another study that included analysis of the adherent stomach microbiota found greater diversity of bacteria in the stomach of yellow grouper compared to other sections of the gut (Zhou *et al.* 2009). The genera *Proteobacterium, Pantoea*, and *Clostridium* were found in all sections of the yellow grouper gut, whereas the less commonly reported phyla Deinococcus-Thermus and Planctomycete were found only in the stomach along with uncultured *Streptococcus* sp. and *Enterobacter*

amnigenus. Interestingly, not all studies have found significant differences between sections. Although included in analysis, no significant differences in adherent community composition in the stomach and intestine were reported for red emperor snapper (*Lutjanus sebae*) (Zhou *et al.* 2009).

When a dietary intervention trial was undertaken on Atlantic cod (*Gadus morhua*) differences in gut microbiota were seen between the different diets, but interestingly, within each diet there was variation in dominant species found in the fore- and midgut, and the hindgut (Ringø *et al.* 2006). Indeed, in fish fed a fishmeal diet, *Psychrobacter* and *Brochothrix* were dominant in the fore- and midgut, while *Carnobacteriaceae* was dominant in the hindgut microbiota. Interestingly, fish fed the soybean meal and the bioprocessed soybean meal diets had *Psychrobacter* dominating throughout the gut. Variation in dominant species in the fore-mid- and hind-gut were also observed in farmed Atlantic salmon (Hovda *et al.* 2007). The fore-gut was dominated by Proteobacteria in the genera *Janthinobacterium*, *Pseudomonas, Acinetobacter*, and *Vibrio*; the mid-gut by the Proteobacteria *Photobacterium phosphoreum* and the genus *Pseudomonas*; while in the hind-gut it was *Vibrio* and *P. phosphoreum* which were present in higher numbers. The differences presented from analyses of different gut segments and gut contents or gut mucus highlights the importance for all studies to report the details of their sample preparation.

Studies investigating the gut microbiota of fish are varied at many levels, including species studied and methods of sample collection and analysis. This can create difficulties when comparing results and extrapolating the true level of diversity. Despite these limitations, results from a comparison which non-uniformly spans a diversity of fish species from over 30 studies revealed the following genera to be the most frequently reported as dominant: *Vibrio* (13 times), *Photobacterium* (8) and *Clostridium* (8) (Table 2.2.2.). In support of these results, a meta-analysis of the gut communities of marine fish revealed that Vibrionales bacteria (which includes the genera *Vibrio* and *Photobacterium*) accounted for 70% of sequence reads (Sullam *et al.* 2012).

Vibrio, a diverse genus of the phylum Proteobacteria, is one of the most important bacterial genera in aquaculture, with both pathogenic and probiotic (health-promoting) species (Vandenberghe *et al.* 2003). *V. anguillarum, V. salmonicida*, and *V. vulnificus* are among the main bacterial pathogens of marine fish and invertebrate species (Austin and Austin 1999). Pathogenic *vibrios* commonly infect larvae and can cause sudden and significant

mortalities. However, it has been hypothesised that many *Vibrio* species are not true pathogens, but in fact opportunistic pathogens whose virulence is accentuated under intensive aquaculture conditions (Thompson *et al.* 2004). *Vibrio alginolyticus*, although sometimes pathogenic (Samad *et al.* 2014; Chen *et al.* 2015), has been shown *in vivo* to work well as a probiotic for Atlantic salmon, protecting against *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii* (Austin *et al.* 1995). An *in vitro* study found that *Vibrio* sp. strain NM 10 had an inhibitory effect against the fish pathogen *Pasteurella piscicida* (Sugita *et al.* 1997). Many *Vibrio* species produce hydrolytic enzymes and in this way, they can act as symbionts assisting in the breakdown of dietary components. Strains have been found to produce amylase (Hamid *et al.* 1979; Gatesoupe *et al.* 1997), lipase (Gatesoupe *et al.* 1997; Henderson and Millar 1998), cellulose (Itoi *et al.* 2006; Sugita and Ito 2006) and chitinase (MacDonald *et al.* 1986) among others (Ray *et al.* 2012).

Photobacterium is also a genus of the phylum Proteobacteria and family *Vibrionaceae*. This luminous bacterium is commonly found on the surface of healthy fish and was originally associated with light-emitting organs, e.g., *Photobacterium angustum, P. leiognathi* and *P. phosphoreum* (Cahill 1990). Initially, it was recognised that surface tubules release these bacteria into the digestive tract of the host (Haygood and Distel 1993). However, since then numerous strains of *Photobacterium* have been found in the GIT of fish species lacking bioluminescent organs (Makemson and Hermosa 1998; Ward *et al.* 2009; Smriga *et al.* 2010). There are also non-luminescent members of the *Photobacterium* genus, such as *P. iliopiscarium* which has been isolated from the intestines of several species of cold-water fish (Onarheim *et al.* 1994; Urakawa *et al.* 1999). Many *Photobacterium* act as mutualistic bacteria in the host gut aiding with chitin digestion (MacDonald *et al.* 1986; Ramesh and Venugopalan 1989; Itoi *et al.* 2006). However, some also produce harmful enzymes such as neuraminidases (Sugita *et al.* 2000). *Photobacterium damselae* is a neuraminidases producer and is a common pathogen for wild and captive fish (Romalde 2002). There are two sub-species of *P. damselae; P. damselae* ssp. *damselae* and *P. damselae* ssp*. piscicida*. The former is associated with skin ulcers, while the latter is the infectious agent of pasteurellosis in fish (Urbanczyk *et al.* 2011).

Clostridium is a very common genus within the phylum Firmicutes. It is a Gram-positive obligatory anaerobe with many pathogenic species. *Clostridium difficile* is a commonly known species of this genus as it is associated with diarrheal disease in humans and animals (Metcalf *et al.* 2011). However, it has not been widely associated with marine fish, though studies

investigating *C. difficile* in fish are limited. One study that did investigate its presence found no *C. difficile* in 107 assorted marine and freshwater fish gut contents (Al Saif and Brazier 1996). It has previously been isolated from freshwater African cichlids (*Nimbochromis venustus*) with the condition known as "Malawi bloat," suggesting that if present it is pathogenic in fish (Dixon *et al.* 1997). *Clostridium botulinum* is a pathogenic species more frequently associated with marine fish. There are six different type strains (A-F). Fish are susceptible to type E and occasionally B (Mazuet *et al.* 2016; Uzalr *et al.* 2016). When 117 intestinal samples from rainbow trout (*Oncorhynchus mykiss*) were analysed for *C. botulinum* type E in Finland, 15% were found positive (Hyytiä *et al.* 1998). Similarly, in a study performed in northern France, the prevalence of *C. botulinum* in marine fish was recorded at 16.6% (Fach *et al.* 2002). Huss and Pedersen (1979) reported that *C. botulinum* was more common for demersal rather than pelagic marine fish and suggested this was as a result of greater interaction with the sediment. It has been noted that fish are not always affected by *C. botulinum* and can be healthy carriers of the spores (Uzal *et al.* 2016).

Clostridium species often work as mutualistic symbionts with marine hosts, especially herbivorous fish (Clements *et al.* 2007; Clements *et al.* 2009). They have been shown to contribute to the host's nutrition, especially by supplying fatty acids and vitamins (Balcázar *et al.* 2006). In southern flounder (*Paralichthys lethostigma*) *Clostridium* along with a number of Gram-negative genera displayed enzyme activities of acid and alkaline phosphatases, C4 and C8 esterases, C14 lipases, arylamidases and glycosidases (Ramirez and Dixon 2003). Beyond this natural symbiosis, some species of *Clostridium*, such as *C. butyricum* have been used successfully as a probiotic in aquaculture, enhancing resistance of rainbow trout to vibriosis (Sakai *et al.* 1995) and stimulating the immune response and improving survival in Japanese flounder (*Paralichthys olivaceus*) (Taoka *et al.* 2006).

Table 2.2.2. Dominant bacterial species isolated from the intestinal tracts of marine fish species at different trophic levels.

Trophic level	Fish species	Dominant bacteria genera	References
Herbivores			
	Butterfish, *Odax pullus*	*Clostridium*	Clements *et al.* (2007)
	Marblefish, *Aplodactylus arctidens*	*Clostridium, Eubacterium desmolans, Papillibacter cinnaminovorans*	Clements *et al.* (2007)
	Parrotfish, *Chlorurus sordidus*	*Vibrio, Photobacterium*	Smriga *et al.* (2010)
	Silver drummer, *Kyphosus sydneyanus*	*Clostridium*	Moran *et al.* (2005)
	Surgeonfish, *Acanthurus nigricans*	Bacteroidetes, non-vibrio Proteobacteria, Firmicutes	Smriga *et al.* (2010)
	Surgeonfish, *Acanthurus sp.*	*Epulopiscium*	Miyake *et al.* (2015)
	Zebraperch, *Hermosilla azurea*	*Enterovibrio,* Bacteroides*, Faecalibacterium, Desulfovibrio*	Fidopiastis *et al.* (2006)
Omnivores			
	Pinfish, *Lagodon rhomboides*	*Clostridium, Mycoplasma*	Ransom (2008)
		Photobacterium, Propionibacterium, Staphylococcus, Pseudomonas, Corynebacterium	Givens *et al.* (2015)
	Long-jawed mudsucker, *Gillichthys mirabilis*	*Mycoplasma*	Bano *et al.* (2007)
Carnivores			
	Atlantic cod, *Gadus morhua*	*Clostridium perfringens*	Aschfalk and Müller (2002)
		Vibrio	Star *et al.* (2013)
	Atlantic halibut, *Hippoglossus*	*Vibrionaceae* (larvae, juveniles), *Photobacterium phosphoreum* (adults)	Verner-Jeffreys *et al.* (2003)
	Atlantic salmon, *Salmo salar*	*Acinetobacter junii, Mycoplasma*	Holben *et al.* (2002)
			Hovda *et al.* (2007)
		Lactobacillus, P. phosphoreum, Lactococcus, Bacillus	
	Blackfin icefish, *Chaenocephalus aceratus*	*Photobacterium*	Ward *et al.* (2009)
	Black rockcod, *Notothenia coriiceps*	*Photobacterium, Vibrio*	Ward *et al.* (2009)
	Bluefish, *Pomatomus saltatrix*	*Vibrio, Pseudomonas,* Enterobacteraceae	Newman *et al.* (1972)
	Gilthead sea bream, *Sparus aurata*	*Pseudomonas*	Floris *et al.* (2013)
	Grass puffer, *Fugu niphobles*	*Vibrio, Pseudomonas, Flavobacterium*	Sugita *et al.* (1989)

Table 2.2.2. Dominant bacterial species isolated from the intestinal tracts of marine fish species at different trophic levels. *Continued.*

Trophic level	Fish species	Dominant bacteria genera	References
Carnivores cont.	Grouper, *Epinephelus coioides*	*Bacillus, Vibrio, Delftia, Psychroacter, Acinetobacter, Pseudomonas*	Sun *et al.* (2009)
	Red drum, *Sciaenops ocellatus*	Mycoplasmataceae	Ransom (2008)
			Givens *et al.* (2015)
	Sea trout, *Salmo trutta trutta*	*Photobacterium, Cetobacterium,* Clostridiaceae*, Vibrio Aeromonas sobria, Pseudomonas*	Skrodenytė-ArbaČlauskiene *et al.* (2008)
	Siberian sturgeon, *Acipenser baerii*	*Cetobacterium somerae*	Geraylou *et al.* (2013)
	Snapper, *Lutjanusn bohar*	*Vibrio*, *Photobacterium*	Smriga *et al.* (2010)
	Southern flounder, *Paralichthys lethostigma*	*Clostridium*	Ramirez and Dixon (2003)
		Clostridium	Ransom (2008)
		Photobacterium, Clostridiaceae, Clostridium	Givens *et al.* (2015)
	Speckled trout, *Cynoscion nebulosus*	*Escherichia coli*	Ransom (2008)
	Striped bass, *Morone saxatilis*	*Aeromonas, Pseudomonas, Vibrio*	MacFarlane *et al.* (1986)
Zooplanktivores			
	Cardinalfish, Apogonidae	*Vibrionaceae* and *Pasteurellaceae*, *Vibrio harveyi*, *Shewanella* sp., *Endozoicomonas* sp.	Parris *et al.* (2016)
	Damselfish, Pomacentridae	*Vibrionaceae* and *Pasteurellaceae*, *Vibrio harveyi*, *Shewanella* sp., *Endozoicomonas* sp.	Parris *et al.* (2016)
	Herring, *Clupea harengus*	*Pseudomonas, Alteromonas*	Hansen *et al.* (1992)
		Pseudomonas, Psychrobacter	Curson *et al.* (2010)
	Pipefish, *Syngnathus scovelli*	Proteobacteria	Ransom (2008)
	Sardines, *Sardinella longiceps*	*Achromobacter, Vibrio, Pseudomonas*	Karthiayani and Mahadeva Iyer (1967)
	Atlantic mackerel, *Scomber scombrus*	*Psychrobacter, Vibrio, Shewanella*	Svanevik and Lunestad (2011a)

5. DIVERSITY OF FISH GUT MICROBIOTA

Studies on the gut microbiota of fish have found substantial intra- and inter-species diversity. Factors which influence this diversity include life stage (Hansen and Olafsen 1999), trophic level (Clements *et al.* 2007), diet (Cordero *et al.* 2015), season (Hovda *et al.* 2012), habitat (Bano *et al.* 2007), captive-state (Dhanasiri *et al.* 2011), sex (Dhanasiri *et al.* 2011), and phylogeny (Miyake *et al.* 2015). A recent meta-analysis investigating the factors affecting the gut microbiota composition of fish reported that trophic level, habitat and possibly host phylogeny are the most likely influencers (Sullam *et al.* 2012). In the following sections, we review the literature thus far relating to the effects of trophic level, season and captive state on the gut microbiota of fish.

5.1. Trophic Level

The influence of diet on gut microbiota is a logical link and has been reported numerous times for an array of species (Claesson *et al.* 2012; Serino *et al.* 2012; Miyake *et al.* 2015; Li *et al.* 2017). Trophic position relates natural diet with evolutionary development and marine fish fill many of these levels. In terms of investigating the relationship of gut microbial composition with trophic level, early studies included flatfish (Liston 1956) and salmon (Yoshimizu and Kimura 1976), however, there was also significant interest in herbivores. The seminal studies of Fishelson (1985) on surgeonfish (*Acanthurus* species) and Rimmer and Wiebe (1987) on sea chub (genus *Kyphosus*) showed for the first time that marine herbivorous fish possessed distinct symbiotic gut microbiota that aided fermentative digestion. Since then, anaerobic bacterial species, frequently of the phylum Firmicutes and class Clostridia, have been repeatedly identified in the digestive tracts of herbivorous fish (Mouchet *et al.* 2012). Within the body of evidence, there has been some replication of species studied, with surgeonfish and sea chub repeatedly investigated (Clements *et al.* 1989; Clements and Choat 1997; Mountfort *et al.* 2002; Moran *et al.* 2005). However, more recently corroborating research found that the microbiota of herbivores was distinct from that of fish with other diets and strains of Firmicutes dominated the gastrointestinal microbial communities of these fish. Published studies that supported these findings worked with a range of different species including brown-spotted spinefoot, (*Siganus stellatus*) butterfish (*Odax pullus*) daisy parrotfish (*Chlorurus sordidus*) dusky parrotfish (*Scarus niger*) marblefish (*Aplodactylus*

arctidens) and zebraperch (*Hermosilla azurea*) (Mountfort *et al.* 2002; Fidopiastis *et al.* 2006; Clements *et al.* 2007; Miyake *et al.* 2015). Clements and associates have driven research within this field and have provided critical reviews of knowledge gained in this area thus far (Clements *et al.* 2009; Clements *et al.* 2014).

Gut microbial communities of fish in other trophic levels have less characteristic dominance when compared to herbivores. However, one study comparing phylogenetically similar benthivore and planktivore freshwater species showed they contained different unique intestinal bacterial communities (Uchii *et al.* 2006). In general, within the marine environment, Proteobacteria, rather than Firmicutes, is often the dominant phylum at the non-herbivorous trophic levels (Miyake *et al.* 2015). *Vibrionaceae, Aeromonas* and *Pseudomonas* are all frequently reported in carnivores, omnivores and (zoo-) planktivores. The gut microbiota of temperate pelagic planktivores such as mackerel (*Scomber scombrus*) and herring as well as tropical planktivores such as pipefish (*Syngnathus scovelli*) sardines (*Sardinella longiceps*) damselfish (*Pomacentridae*) and cardinalfish (*Apogonidae*) have all been studied (Karthiayani and Mahadeva Iyer 1967; Hansen *et al.* 1992; Ransom 2008; Svanevik and Lunestad 2011b; Parris *et al.* 2016). The dominant species reported were Gram-negative bacteria such as *Vibrio, Pseudomonas, Psychrobacter, Achromobacter, Shewanella, Alteromonas, Endozoicomonas, Vibrionaceae* and *Pasteurellaceae* (Table 2.2.2.). Two studies that looked at omnivore species; long-jawed mudsucker (*Gillichthys mirabilis*) and pinfish (*Lagodon rhomboids*) reported *Mycoplasma* spp. as the dominant bacteria (Table 2.2.2.) (Bano *et al.* 2007; Ransom 2008).

We compared 17 different published studies, which provided data on dominant gut bacteria in 16 carnivorous species and found *Vibrio* (9 times) and *Photobacterium* (7) were the most frequently reported. *Pseudomonas* was reported six different times while *Clostridium* was found in three species by five different studies. Finally, *Aeromonas, Cetobacterium, Bacillus, Mycoplasma* and *Acinetobacter* were reported twice (Table 2.2.2.). Of these *Aeromonas, Photobacterium, Pseudomonas* and *Vibrio* have all been identified as fish gut microbiota that might aid digestion (Ray *et al.* 2012). *Vibrio* spp., *Enterobacter* spp., *Pseudomonas* spp. and *Aeromonas* spp. isolated from marine fish GIT have been found to produce proteases while these bacteria along with *Photobacterium* spp. have also been reported to produce chitinases (Hamid *et al.* 1979; MacDonald *et al.* 1986; Gatesoupe *et al.* 1997; Hoshino *et al.* 1997; Itoi *et al.* 2006). Knowledge of the principle composition of fishes'

gut microbiota and understanding the role they play in digestion and whole-body function is critical. This is especially important as new species continue to enter the aquaculture sector and diet manipulation becomes common practise as a means to improve health and performance. The use of shotgun sequencing and transcriptome analysis in future studies will be imperative to meet this goal but it will be essential that such studies distinguish between the residential species and those which have been ingested.

5.2. Season

Several reviews have highlighted seasonal variation and temperature changes as a defining parameter for fish gut microbial composition (Nayak 2010; Sullam *et al.* 2012; Ringø *et al.* 2016). However, the majority of studies reporting this have been conducted on freshwater fish (Sugita *et al.* 1983; Sugita *et al.* 1987; Macmillan and Santucci 1990; Spanggaard *et al.* 2000; Al-Harbi and Uddin 2004; Hagi *et al.* 2004; Naviner *et al.* 2006). Changes in total bacterial abundance have been reported, with peaks in summer and autumn months (Macmillan and Santucci 1990; Al-Harbi and Uddin 2004) as well as variations in dominant species (Hagi *et al.* 2004).

Historically, seasonal trends were reported in total bacterial counts recorded from gut samples from skate (*Raja* sp.) and lemon sole (*Pleuronectes mimocephalus*) plated on seawater agar (Liston 1956). This study suggested that changes in plankton availability influenced the gut bacterial composition in fish. To the best of our knowledge, the first study to directly investigate seasonal variation in gut microbiota in marine fish was by Hovda *et al.* (2012). The gut microbiota of adult Atlantic salmon was analysed between August and June the following year, using 16S rRNA DNA sequencing. The water temperature varied between 5.5 and 18.8 ^{o}C during the experimental period. Although some bacterial species were only recorded at some of the sample time points, overall the variation reported was not statistically significant. Contrarily, a more recent study on salmon did find a relationship between seasonal water temperature changes and shifts in gut microbial composition (Neuman *et al.* 2016). In this study, increasing temperatures (up to 21 ^{o}C) were associated with a disappearance of lactic acid bacteria (LAB) and *Acinetobacter* spp. and an increase in *Vibrio* spp. The loss of protective LAB and an increase in potentially virulent *Vibrio* spp. could have a negative impact on host health and has the potential to become an important issue

with sea temperatures rising and stocks of wild salmon decreasing. Further research is required to determine the effects of seasonal variation and temperature changes on the gut microbiota of marine fish.

5.3. Wild vs. captured

Captive breeding and rearing of fish commonly involve the manipulation of multiple factors, including environment, social interaction and diets. Unnatural stocking densities and increased stress levels can lead to spread of disease, a major problem for the aquaculture industry (Verschuere *et al.* 2000).Within the sector, antibiotics have been used liberally to clear bacterial infections and even prophylactically to compensate for shortfalls in substandard rearing conditions (Cabello 2006). The result of this is resistance development in aquaculture pathogens (Defoirdt *et al.* 2011), and reduction of microbial gut diversity in aquaculture species (Navarrete *et al.* 2008). Today, as regulations on the use of antibiotics in aquaculture are becoming more stringent in many countries, research into alternative methods of disease control are being prioritised. However, the aquaculture industry continues to expand, and such regulations are still lax in many areas on a global scale. Assessment of the level of use and the impact of antibiotics on aquaculture and wild fish is crucial. This is an important topic that is worthy of a full review in its own right. See the following reviews for more in-depth discussion; Romero *et al.* (2012), Henriksson *et al.* (2017), and Lozano *et al.* (2018).

Artificial diets and increased food intake levels, often with concomitant increases in stress, can cause alterations in the microbiota in fish GIT (Clements *et al.* 2014). A frequently cited study that clearly depicts this relationship reports the changes in the gut microbiota of wild Atlantic cod after captive rearing (Dhanasiri *et al.* 2011). In this study, total counts of bacteria did not vary significantly but the diversity of bacterial species reduced notably after 6 weeks of artificial feeding. However, the study omits information on specific bacteria that are associated with the wild and subsequent captive states. Contrarily, when the gut microbiota of wild and pen-reared Atlantic salmon were compared, farmed fish had a greater microbial diversity (Holben *et al.* 2002). Interestingly, a novel *Mycoplasma* phylotype was found to dominate in wild Atlantic salmon and pen-reared fish in Scotland, whereas the farmed fish in Norway were dominated by *Acinetobacter junii*. The farmed fish in the two

locations were fed different diets. Another study looking at changes in gut microbiota of salmon throughout the life cycle observed that all stages were dominated by Proteobacteria and were enriched for Tenericutes (genus *Mycoplasma* especially; (Llewellyn *et al.* 2016). Taken together, these studies suggest the presence of a core microbiota that can persist often in spite of changing factors. Other studies have also reported results to support this "core microbiota" hypothesis (Roeselers *et al.* 2011). In the fish model species zebrafish, it was shown that there were significant similarities in the gut microbiota found in fish collected recently from their natural habitat and those reared for generations in lab facilities. However, also observed were variations correlated to lab facility and historical connections between these different sites (Roeselers *et al.* 2011).

One of the most egregious alterations commonly encountered by farmed fish is the increasing inclusion of plant ingredients into carnivorous diets. The ability of carnivorous fish to adaptively modulate digestive functions to meet changes in diet composition is limited (Buddington *et al.* 1997). Feed efficiency, growth rates, whole body composition of fish and nitrogen retention were significantly, negatively affected when 80% or more fishmeal was replaced by plant proteins in diets fed to juvenile turbot (*Psetta maxima*; Fournier *et al.* 2004). Similar results were reported in a study performed on red sea bream (*Pagrus major*) whereby the experimental diet with low fishmeal and high plant protein levels caused significant reductions in feed conversion and protein efficiency ratio, digestibility of protein and disease resistance against *Edwardsiella tarda* (Khosravi *et al.* 2015). Studies focussed on plant protein digestion in salmonids predominate among the published literature. Addition of plant-based proteins into salmonid diets has caused numerous intestinal disorders (Urán *et al.* 2008; Penn *et al.* 2011; Sahlmann *et al.* 2013). These intestinal disorders are frequently reported in conjunction with alterations in the gut microbiota (Bakke-McKellep *et al.* 2007; Green *et al.* 2013). In Atlantic salmon, soybean meal-induced enteritis was accompanied by increased numbers and diversity of gut bacteria, although numbers of LAB were reduced compared to fish on a fishmeal-based diet (Bakke-McKellep *et al.* 2007). Similarly, Green *et al.* (2013) found that salmon fed soy protein concentrate experienced intestinal disorders at high seawater temperatures and coincidently experienced increased bacterial diversity which included bacteria not normally associated with marine fish (*Escherichia* and *Propionibacterium*). Gut microbial changes related to plant-protein diets have also been recorded in other carnivorous fish species. In Atlantic cod, Gram-negative bacteria *Chryseobacterium* spp. and

Psychrobacter glacincola, and Gram-positive bacteria belonging to *Carnobacterium*, were dominant in the GIT of fish fed soybean meal while fish fed fishmeal were dominated by Gram-positive bacteria of the genera *Brochothrix* and *Carnobacterium* only (Ringø *et al.* 2006). Now that a link between certain plant ingredients, changes in gut microbiota composition and intestinal disorders are recognised, concerted efforts are being made to reduce the negative impacts of these ingredients, often through supplementation and further modification of the compound diets (Barrows *et al.* 2008; Krogdahl *et al.* 2010).

Changes in gut microbiota composition attributed to captive-state have also been reported in freshwater fish (Bucio *et al.* 2006) as well as other marine animals (Nelson *et al.* 2013) and are now generally accepted. However, knowledge of the gut microbiota in wild marine fish requires more attention to provide a baseline for comparative purposes to better understand the effects of captive rearing.

6. MANIPULATION OF THE FISH GUT MICROBIOTA

The innate link between a host's microbial community and its health status is recognised in humans and other animals and much research is now directed toward methods to manipulate these microbial communities to boost host health. Fish have not been omitted from this area of nutrition and with the growth of the aquaculture industry, there has been a growing interest in the manipulation of fish gut microbiota to improve welfare and nutrition. The principle methods of gut microbial manipulation have included the alteration of dietary proteins and lipids, as well as the addition of probiotics and prebiotics in the diet.

6.1. Proteins

Proteins, the building blocks of the body, are involved in a plethora of chemical pathways and bodily functions. The source (Desai *et al.* 2012), quantity (Geurden *et al.* 2014) and chemical structure (Kotzamanis *et al.* 2007) of proteins can influence gut health and microbial composition. In Atlantic salmon dietary protein quantity has been shown to alter gut microbiota. A recent study reported an association between reduced protein levels in the diet and a more divergent microbial community structure in the gut (Zarkasi *et al.* 2016). Peptides and glycopeptides, released through hydrolytic digestion modulate the condition and activity of the intestinal cells as well as the residing microbiota (Świątecka *et al.* 2012). Altering dietary protein by providing protein hydrolysates can directly and indirectly change the hosts gut microbial community. Introduction of short peptides to the diet can directly manipulate gut microbial composition by providing suitable substrates for bacteria thus encouraging proliferation (Kotzamanis *et al.* 2007; Delcroix *et al.* 2015). Certain peptides can exhibit antimicrobial activity and thus help to protect against pathogenic bacteria (Sila *et al.* 2014). Indirectly, they are thought to result in rapid absorption of amino acids, decreasing splanchnic extraction, causing higher systemic amino acid levels (Manninen 2009; Egerton *et al.* 2018). Single amino acids play an important role in immune defence, contributing to the synthesis of antibodies and controlling key immune regulatory pathways (Kiron 2012). Improved immunity, often associated with dietary protein hydrolysates, can allow for the reduction in pathogenic gut microbiota (Tang *et al.* 2008; Bui *et al.* 2014; Khosravi *et al.* 2015). The source and the degree of hydrolysis of proteins in fish diets have been reported to alter gut microbiota in larvae. Changes in culturable bacteria, especially *Vibrio* spp., were reported with

seabass (*Dicentrarchus labrax*) larvae (Kotzamanis *et al.* 2007). Delcroix *et al.* (2015) also reported significant differences in gut microbiota related to diet but did not provide details of composition.

6.2. Lipids

Fat or oil source and composition is an area of great interest in human nutrition. A recent seminal study used a rat model to show how fat type (saturated animal lard vs. polyunsaturated fish oil) altered the gut microbiota and in turn affected white adipose tissue (WAT) inflammation (Caesar *et al.* 2015). Lipids are important macronutrients in the diet of fish. Investigations of dietary lipids have been long-standing. The level of lipid inclusion has been examined (Lesel *et al.* 1989; Ringø and Birkbeck 1999b) and Lesel *et al.* (1989) found that increasing lipid concentrations resulted in a more diverse gut microbial community. More importantly, for the aquaculture industry, the substitution of fish oils for different dietary plant oils has also been studied (Ringø *et al.* 2002; Montero *et al.* 2010). All-natural plant oils are deficient in marine polyunsaturated fatty acids; arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid (Merrifiel *et al.* 2011). Ringø *et al.* (2002) found differences of the aerobic gut microbial communities of Arctic charr (*Salvelinus alpinus* L.) fed soybean, linseed or marine oils. This study and others (Hardy 1997; Lødemel *et al.* 2001) have shown that replacement of fish oils with plant oils can actually improve fishes' resistance to pathogenic bacteria, for which Ringø *et al.* (2002) suggests the associated gut microbial change plays a role. Further research is needed on this topic to confirm the effects of lipid source, composition and concentration on fish gut microbiota. Furthermore, long-chain polyunsaturated fatty acid synthesising bacteria have been discussed in the literature for over two decades and it is reported that they have mostly been isolated from marine sources such as seawater, fish, and sediments (Nichols and McMeekin 2002; Yoshida *et al.* 2016). *Shewanella* sp. along with *Vibrio* sp. are the major PUFA-producing bacterial species isolated from the GIT of fish and invertebrates (Monroig *et al.* 2013). Research into potential use of such bacteria for probiotic purposes would be a novel and interesting route of investigation for fish health and nutrition.

6.3. Probiotics

Probiotics are defined as 'live microorganisms which, when administered in adequate amounts, provide a health benefit to the host' (WHO and FAO 2006). Their use in aquaculture, as an alternative to antibiotics, rose significantly as legislation was introduced that restricted the widespread use of chemicals in animal rearing (Abelli *et al.* 2009). Gram-positive and Gram-negative bacteria, bacteriophages, microalgae and yeasts have all been tested as potential probiotics in fish (Akhter *et al.* 2015). Some of the most frequently investigated probiotics include LAB *Bacillus, Lactococcus, Shewanella*, and *Aeromonas* genera (Hagi *et al.* 2004; Burr *et al.* 2005; Merrifield and Carnevali 2014). In a recent review, Carnevali *et al.* (2017) listed 61 published studies that investigated the administration of probiotics to teleosts. In conjunction with the manipulation of the gut microbial composition, many studies have reported an increase in growth rates (Gatesoupe 1991; Bagheri *et al.* 2008; Lobo *et al.* 2014) and modulation of immune status (Balcázar *et al.* 2006; Huang *et al.* 2014; Cordero *et al.* 2015). Thus far, trials have mostly been performed on larvae and juveniles from which positive effects in the intestinal mucosal cells and stimulation of the innate immune response have been reported (Cerezuela *et al.* 2011; Abid *et al.* 2013). However, in aquaculture the successful administration of probiotics can be difficult. Issues reported include low viability of the bacteria during processing and storage, loss from leaching in the water during feeding, as well as problems related to feed handling and preparation (Merrifield *et al.* 2010). Despite this, when successfully administered, probiotics have been found to reduce the cost of fish farming through improvements in fish welfare and nutrition (El-Haroun *et al.* 2006).

6.4. Prebiotics

Contrarily to probiotics, prebiotics do not introduce novel microbiota into the intestinal tract, but rather are defined as 'substrates that are selectively used by host microorganisms conferring a health benefit' (Gibson *et al.* 2017). As a result, these indigestible food ingredients have been shown to enhance immune response (Torrecillas *et al.* 2007), improve nutrient uptake (Bongers and van den Heuvel 2003) and increase growth and feed conversion ratios (Adel *et al.* 2016). There are also fewer difficulties compared to probiotics in successfully administering these supplements. The principle prebiotics used for fish are fructo-oligosaccharides, short-chain fructooligosaccharides, oligofructose,

mannanoligosaccharides, trans-galactooligosaccharides, inulin, galactooligosaccharides, xylooligosaccharides, arabinoxylooligosaccharides and isomaltooligosaccharides (Ringø *et al.* 2016). Results of prebiotic feeding studies vary considerably (Burr *et al.* 2010; Zhou *et al.* 2010; Torrecillas *et al.* 2014) and it appears likely that success will be supplement and dose-dependent, with considerations for time of supplementation, culture conditions, fish species and age required (Torrecillas *et al.* 2014). Prebiotics are sometimes used in conjunction with probiotics, creating a nutritional mixture (synbiotic) that can provide enhanced benefits for the host (Cerezuela *et al.* 2011). This enhanced effect was initially hypothesised as probiotics are mainly active in the small intestine, while prebiotics influence the microbiota of the large intestine in humans (Gibson and Roberfroid 1995). Some studies have reported supportive results to suggest an enhanced effect of synbiotics over prebiotics or probiotics alone (Rodriguez-Estrada *et al.* 2009; Mehrabi *et al.* 2012). However, there has been some disparity within the published studies (Ai *et al.* 2011; Geng *et al.* 2011).

The use of probiotics and prebiotics in aquaculture is a fast-growing area and research is building an understanding of the mechanistic pathways within which they work. For recent comprehensive reviews on this topic see Cerezuela *et al.* (2011), Dimitroglou *et al.* (2011), Torrecillas *et al.* (2014), Song *et al.* (2014), Akhter *et al.* (2015), Ringø *et al.* (2016), and Carnevali *et al.* (2017).

7. CONCLUSION

Similar to mammals, the gut microbiota of fish can be recognised as an organ, in itself responsible for key physiological functions which aid health maintenance of its host. Knowledge of its composition and exact functional role in health and disease is vital given the environmental changes to which fish are being exposed, particularly in light of the growth of the aquaculture industry and rising sea temperatures as a result of climate change.

The literature on the gut microbiota of marine fish thus far has provided an understanding of many areas and we now appreciate the mechanisms of colonisation and development of the fish gut microbiota. Earlier studies had suggested that bacterial levels in the fish gut were low (Yoshimizu and Kimura 1976), while recent studies, with the help of advanced molecular techniques including next generation sequencing technologies, have painted a different view (Zarkasi *et al.* 2014) and numbers have been shown to reach as high as 10^9 cfu g^{-1} in gut content of particular species.

It has been reported that 90% of fish intestinal microbiota studied to date are composed of Proteobacteria, Bacteroidetes and Firmicutes (Ghanbari *et al.* 2015). However, within these phyla, studies reporting gut microbiota composition have generally conveyed conflicting results and this is undoubtedly a feature of the diversity which exists amongst fish. Such diversity in results can pose difficulties in extrapolating real and meaningful trends and correlations between gut microbial composition and the factors that shape it. Despite this, studies generated to date have enabled us to infer certain conclusions such as the dominance of particular genera where the genus *Vibrio* appears to be a key member followed closely by *Photobacterium* and *Clostridium*. However, further studies are warranted to confirm such inferences. Efforts to improve and standardise sample collection, including differentiating between allochthonous and autochthonous bacteria, and subsequent analysis should greatly benefit inter-study comparisons and add strength to the data reported. Undoubtedly next generation sequencing technologies will help this enormously, providing more comprehensive datasets.

Diet and trophic level have presented as clear influencing factors of fish gut microbial composition. It has been shown that *Clostridium* is linked to an herbivorous diet while *Vibrio* and *Photobacterium* are commonly found in carnivores. Seasonal changes and the associated changes in water temperature and captive rearing have also been shown to influence

microbiota composition and certain studies have cited the detrimental impact of each. In this regard, strategies which enable the manipulation of gut microbiota composition toward that of a healthy microbiota are essential. Probiotics and prebiotics are at the forefront of this but perhaps one of the greatest impediments is the lack of baseline compositional data from healthy wild fish in their natural environments. Thus, an increased focus toward collecting such data is essential if dietary manipulation strategies are to be of full benefit. Inarguably, the need to better understand the innate relationship between gut microbiota and their fish hosts is the ultimate goal. Some excellent initial work on the role and mechanistic pathways of gut microbiota has been produced (Ringø *et al.* 2010; Ray *et al.* 2012). However, gaining a greater understanding of the specific effects of particular microbes and their associated components on host health will improve our ability to manipulate and fortify fish gut microbial communities to enhance fish health and aquaculture productivity. The use of transcriptomics will be important in this future research.

There are a number of important topics in this area that would benefit from further research in the future. Firstly, producing baseline data on the gut microbiota of wild populations, which includes domains beyond just bacteria should be prioritised. Investigations into the potential effects of on-coming climate change including changes in water salinity, acidity and temperature on the gut microbiota of fish will be important. The other area that will continue to be prioritised is diet manipulation. Finding diets that are sustainable and also benefit the fish in terms of nutrition and health is imperative for the aquaculture industry. Throughout these studies, the role of the gut microbiota will need to be considered. Finally, the supply of marine lipids is becoming an inhibitory factor for the aquaculture industry. In this review, we have highlighted two interesting areas of research related to this which are worthy of further research. Firstly, initial reports linking dietary plant oils to pathogenic resistance and secondly, the formative research on PUFA-producing bacteria that could potentially play an important role in meeting the future demands for marine lipids. Although researchers working in this field have significantly expanded our knowledge on this topic there is still great scope for further research. Data collection from wild populations, laboratory experiments and work within aquaculture will all be important contributors.

Acknowledgements

We would like to gratefully acknowledge the funding of this work by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. via the IRC Enterprise Partnership Scheme.

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Chapter 3

The proximate, protein and lipid composition of three commercially important marine fish; blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*) and Atlantic herring (*Clupea harengus*)

Under review: *Irish Journal of Agriculture and Food Research* (Jan 2020)

ABSTRACT

This study presents data from in-depth proximate compositional analyses of three fish species; blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*), and Atlantic herring (*Clupea harengus*). These fish contained significant amounts of high-quality protein (16 – 17%), lipids (4 – 11%) and minerals (2 – 6% ash). The proteins, particularly from boarfish, had close to optimum amino acid profiles for human and fish nutrition. These fish compared highly with other fish species in terms of total lipids and relative concentration of DHA and EPA omega-3 fatty acids (11.8 – 13.3% and 5.9 – 8.1% of triglycerides (TG) and 24.6 – 35.4% and 5.8 – 12.0% of phospholipids (PL), respectively). Atlantic herring had the highest lipid content of the three fish and was found to contain high levels of PL PUFA and omega-3 fatty acids. Boarfish and blue whiting both had higher relative abundance of TG PUFA and omega-3 fatty acids compared to Atlantic herring, but these were below average compared to results for marine fish from other studies. Minerals detected in the fish included calcium (272 - 1520 mg $100g^{-1}$), phosphorus (363 - 789 mg $100g^{-1}$), iron, magnesium, potassium, selenium, sodium and zinc. Boarfish had the highest ash fraction and also the highest levels of each mineral, except potassium. Atlantic herring had considerably lower mineral content compared to the other two species and levels detected were also lower than reported in previous published studies. Heavy metals (arsenic, cadmium, mercury, lead, copper and zinc) were also tested and levels in the three fish species were significantly below maximum allowable limits (MAL) for all elements except zinc. Arsenic levels, which ranged from 1.34 – 2.44 mg kg^{-1}, were also close to the MAL. The data outlined here will be useful for guiding product development. Future studies would benefit from considering catch season, sex and developmental stage of the fish.

1. INTRODUCTION

Marine pelagic fish constitute critically important resources globally. Lower-tropic level (LTL) pelagic species or 'forage fish' account for over 30% of global fish landings (Smith *et al.* 2011). LTL pelagic stocks (Atlantic herring, *Clupea harengus*, blue whiting, *Micromesistius poutassou*, boarfish, *Capros aper*, Atlantic mackerel, *Scomber scombrus,* and Atlantic horse mackerel, *Trachurus trachurus*) have always been an important component of the Irish fishing industry. While they dominate Irish landings in terms of tonnage, only mackerel and horse mackerel currently contribute considerably to the value of landings (Gerritsen and Lordan 2014).

The level of demand for Atlantic herring, blue whiting and boarfish for direct human consumption does not match the volume in which they are landed. Therefore, these fish are commonly sold at low prices for reduction to fishmeal and low-grade fish oil for animal feeds (Neiland 2007; Alder *et al.* 2008; Egerton *et al.* 2017). However, researchers are investigating alternative processing methods, products and uses which could add value to these fisheries. Thorough and reliable knowledge of the compositional make-up of the raw material is imperative for processing and product development. Thus far, the published research in this area has principally focussed on single fraction compositional studies rather than providing complete, in-depth proximal composition data.

In the 1960s, at least 90% of all fish oils were produced from different species of herring (Lambertsen and Brækkan 1965). Therefore, there is a knowledge base of lipid composition for this species. However, much of the work has focussed on Pacific (Ackman and Eaton 1966; Huynh *et al.* 2007) or Baltic herring (Aro *et al.* 2000; Szlinder-Richert *et al.* 2010), or else Atlantic herring fished off the North American coast (Ackman *et al.* 1967; Lane *et al.* 2011).Furthermore, there has been much less interest in the protein fraction of Atlantic herring, with only a few published studies investigating protein hydrolysates derived from this species (Sathivel *et al.* 2003; Beaulieu *et al.* 2009; Pampanin *et al.* 2012).

Contrarily, research on processing and product development for blue whiting and boarfish have focussed on the protein fraction with little or no research on their lipid fraction (Huidobro and Tejada 1995; Pérez-Mateos *et al.* 1997; Fernández-Martín *et al.* 1998; Cudennec *et al.* 2012; Nobile *et al.* 2016; Ojha *et al.* 2016; McLaughlin *et al.* 2017; Crowe *et al.* 2018; Egerton *et al.* 2018; Parthsarathy *et al.* 2019). We could only find two pieces of work relating to boarfish lipids; a magazine article briefly investigating the omega-3 contents of

boarfish (Gormley 2015) and a peer-reviewed article reporting the lipid composition and fatty acids profile in selected fishmeals, of which boarfish was an ingredient (Mika *et al.* 2016).

Mineral content can sometimes be overlooked in research on nutrient extraction from fish. Thus far, there has been no published research on the mineral content in boarfish. Whereas, the mineral content of blue whiting and herring have been reported in a number of studies. Blue whiting was one of a selection of marine fish sampled in two studies investigating mineral content of fish from Norwegian and Mediterranean seas (Toppe *et al.* 2007). The Norwegian study also included Baltic herring. Other studies that have reported mineral content of herring have taken fish from off the coast of Alaska (Sathivel *et al.* 2003), Cameroon (Tenyang *et al.* 2014) and from the Baltic Sea (Tahvonen *et al.* 2000; Marmon and Undeland 2010). Location of fish development is a known factor that can influence mineral content (Martínez-Valverde *et al.* 2000). Despite this, we have been unable to find any studies reporting the mineral content of these species fished in the Northeast Atlantic region.

This study compares the proximate composition (protein, lipid, moisture and ash) of the LTL pelagic species; blue whiting, boarfish and Atlantic herring (from here on referred to as herring unless differentiation from Pacific herring is required) from Irish landings fished in the Northeast Atlantic region. It presents results from in-depth analysis of the protein, lipid and mineral compositions, providing information that can be used to guide future processing and product development. The chemical and nutritional compositional data generated in this study, which includes fatty acid profiles as analysed by gas chromatography, will benefit the seafood industry but are also important information that could be used for stock quality and prey assessment (Lawson *et al.* 1998; Hartman and Margraf 2008).

2. MATERIALS AND METHODS

2.1. Materials

Two deliveries of blue whiting, boarfish and herring were kindly donated by Biomarine Ingredients Ireland Ltd. The fish were caught off the north-west coast of Ireland and immediately frozen at sea. They were transported frozen, on ice to the laboratory and were stored at −20 °C until required. Upon thawing, whole fish were homogenized (Robot Coupe blixer 2, Stephens Catering Equipment Co. Ltd., Ireland) individually to create a uniform mince of each fish. The mince was stored in aliquots at -20 °C and defrosted as required prior to analysis.

2.2. Chemical reagents

Cyclohexane, hexane, heptane, methanol, propan-2-ol, formic acid, hydrochloric acid, 2 M hydrochloric acid in methanol and 25% sodium methoxide were purchased from Sigma Aldrich (Dublin, Ireland). Diethyl ether was purchased from Fisher Scientific (Dublin, Ireland). Triglyceride (TG) standard trinonadecanoin glyceride (C19:0) (part no. T-165), fatty acid methyl ester (FAME) standard methyl nonadecanoate (C19:0) (part no. N-19M) and FAME standard mix containing C10:0 to C24:1 methyl esters (part no. GLC 462) were purchased from Nu-Chek Prep Inc. (Waterville, MN, USA). Phospholipid (PL) standard 1,2-dinonadecanoyl-sn-glycero-3-phosphocholine (C19:0 PC) (part no. 850367P-AVL) was purchased from Stratech (Suffolk, England). Aminopropyl cartridges (500 mg, part no. 12102041) were obtained from Agilent Technologies (Little Island, Cork, Ireland).

2.3. Proximate composition

Proximate composition was determined for individual whole wet fish (n = 30). Moisture content was determined gravimetrically after drying at 105 °C for 24 h. Ash content was measured by incinerating the dried samples overnight in a furnace at 550 °C. Total nitrogen content was determined by the macro-Kjeldahl method and crude protein content was estimated by multiplying total nitrogen content by 6.25. Lipid content was determined gravimetrically after extraction following a modified Bligh and Dyer method (Smedes 1999). Moisture and ash analyses were performed in triplicate, while protein and fat analyses were performed in duplicate.

2.4. Protein amino acid composition

A single, pooled sample (*n* = 6) per species of whole homogenised fish were hydrolysed in 6 N hydrochloric acid at 110 °C for 23 h and total amino acid content was determined for the resulting hydrolysates according to McDermott *et al.* (2016). Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column.

2.5. Lipid composition

Total lipids were extracted from whole homogenised fish samples following a modified Bligh and Dyer method (Smedes 1999). Lipid classes (TG, free fatty acids (FFA), PL and glycolipids (GL)) were separated by solid-phase extraction following the methods described in O'Callaghan *et al.* (2016) with some modifications. Briefly, 500 mg aminopropyl columns (Agilent Technologies Ltd.) were preconditioned with 10 mL of heptane. The lipid extract was applied to the column, a vacuum was applied, and the TG were eluted. Remaining TG were removed using 10 mL of 20% diethyl ether/hexane (vol/vol). Next, FFA were eluted using 5 mL of 2% formic acid/diethyl ether (vol/vol) followed by GL and PL using 5 mL of acetone/isopropanol (1:1, v/v) and 5 mL of methanol, respectively. The separated lipid classes were dried down under a flow of nitrogen and weighed. Subsequently, the entire PL extract was re-dissolved in hexane (500 µL) and an aliquot (200 mg) of TG was re-dissolved in hexane (25 mL).

The methyl esters and standard curve for FAME analysis, along with in-run quality control samples, were prepared using Agilent 7696A Sample Prep Workbench (Agilent Technologies Ltd.). TG and PL extracts were trans-methylated by mixing with 0.5 M sodium methoxide in anhydrous methanol (24:1, v/ v) at 25 ^{o}C (Carvalho and Malcata 2005). The reaction was neutralised with 0.5 M hydrochloric acid in methanol and trans-methyl esters in the top layer were transferred to amber glass vials for gas chromatography (GC) analysis. Each sample included the internal standard nonadecanoic acid (C19:0). FAMEs were analysed on an Agilent 7890B gas chromatograph, equipped with a flame ionization detector, GC80 autosampler and a multimode inlet injector (Agilent Technologies Ltd.). The column was a Chrompack CP Sil 88 column (100 m x 0.25 mm internal diameter, 0.25 µm phase thickness,

Chrompack, JVA Analytical), helium was used as the carrier gas, which was held at a constant flow of 1.2 mL min^{-1}. The injector was held at 300 °C for the entire run and was operated in split/spitless mode using a split of 1:20. The inlet liner used was a wool packed liner (cat. no. 8004-0118, Agilent Technologies). The oven cycle was programmed initially at 80 °C for 8 min followed by an increase by 8.5 °C min^{-1} to a final column temperature of 200 °C. Total run time was 72 min. The flame ionization detector (FID) was operated at 300 °C. Peak integration was performed using the Agilent OpenLAB Chemstation software (Version A.01.06.111).

Identification of individual FAME was achieved by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA) and quantification against the internal standards. Results are expressed as g 100 g^{-1} of total detected FAME.

2.6. Mineral composition

Mineral and heavy metal analysis was completed on a single pooled sample (n = 6) of whole blue whiting, boarfish and herring, respectively (ALS Life Sciences, Laois, Ireland). Briefly, all samples were homogenised and mineralized by acids and hydrogen peroxide prior to analysis. Calcium, magnesium, phosphorus, potassium, sodium, iron and zinc were determined by atomic emission spectrometry with inductively coupled plasma and stoichiometric calculations of compounds concentration from measured values. Selenium, arsenic, cadmium, copper and lead were similarly determined using mass spectrometry. Mercury was determined by atomic absorption spectrometry.

2.7. Statistical Analysis

Statistical analyses were carried out using GraphPad Prism version 7.02. Proximate composition data (n = 30) were submitted to Analysis of Variance (ANOVA) and pairwise comparisons were conducted by Tukey's test. Pearson’s correlation was used to investigate the relationship between total moisture and lipids of the fish. Lipid composition data (n = 3) was analysed in duplicate and submitted to Analysis of Variance (ANOVA) and pairwise comparisons were conducted by Tukey's test. Significance level was determined at the 95% probability level.

3. RESULTS

3.1. Proximate composition

The proximate composition of 30 fish from each species was analysed. Significant differences in the percent of whole-body weight were found in moisture, ash, protein and lipid content (Figure 3.1.). Protein content was the most similar fraction in the three fish species. Protein content was lowest in boarfish (15.9 ± 1.4%) which was found to be significantly lower than blue whiting protein content (16.9 ± 1.4%; $F(2, 87) = 4.094$, $p < 0.05$) but not herring (16.6 ± 1.1%).

Herring (11.2 ± 3.0%) had a significantly higher lipid content compared to boarfish (7.3 ± 3.2%), which in turn had a significantly higher lipid content to blue whiting (3.9 ± 1.4%). Comparing the total fat content of these three species with that of other marine fish reported in the literature, Atlantic herring had the highest total lipid content while boarfish and blue whiting were fattier than 89% and 69% of fish, respectively (Table 3.1.).

Moisture content was highest in blue whiting (77.3 ± 1.9%) and lowest in herring (69.3± 7.2%). Herring also showed the greatest variation in moisture content between individuals (range: 55.0 to 84.0%). A negative correlation between moisture content and lipid content of individuals in all three species was clearly observed ($R^2 = 0.6623$, $p < 0.001$; Figure 3.2.).

Finally, boarfish was found to have the highest ash content (6.0 ± 1.1%). This was significantly higher than blue whiting (3.6 ± 0.5%) which in turn was significantly higher than the ash content of herring (2.1 ± 0.5%; $F(2, 87) = 181.8$, $p < 0.001$).

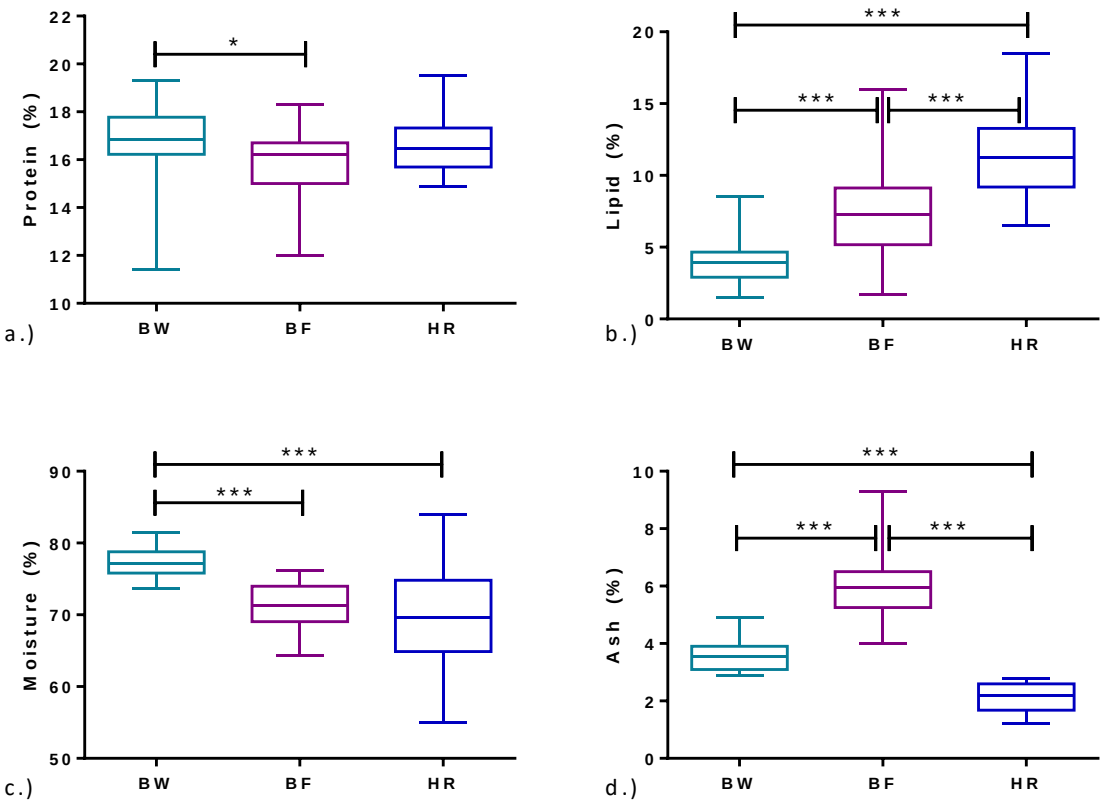

Figure 3.1. The proximate composition (protein, lipid, moisture and ash) of blue whiting (BW), boarfish (BF) and herring (HR) as percent of whole-body wet weight (*n*=30).

Table 3.1. Total lipid content (% wet weight) of important marine fish in the Mediterranean, Aegean and Black Seas and the Pacific Ocean as reported in three published research articles as well as the results from this study for blue whiting, Atlantic herring and boarfish.

Fish species	**% wet weight**
Merluccius productus (Pacific hake)	0.73 ± 0.29[b]
Solea (Common sole)	0.74 ± 0.05[a]
Theragra chalcogramma (Walleye pollock)	0.79 ± 0.17[b]
Scorpaena scrofa (Red scorpion fish)	0.87 ± 0.04[a]
Scopthalmus maeticus (Turbot)	1.30 ± 0.12[a]
Trachurus mediterraneous (Scad)	1.37 ± 0.25[a]
Pagellus erythrinus (Pandora)	1.67 ± 0.02[a]
Sarpa salpa (Salema)	1.67 ± 0.11[c]
Gobius niger (Black goby)	1.92 ± 0.05[c]
Mugil cephalus (Mullet)	2.09 ± 0.07[a]
Zoosterisessor ophiocephalus (Grass goby)	2.13 ± 0.13[c]
Dicenthrarcus labrax (Seabass)	2.33 ± 0.10[c]
Spicara smaris (Picarel)	2.84 ± 0.20[c]
Atherina boyeri (Sand smelt)	2.94 ± 0.16[c]
Hypomesus pretiosus (Surf smelt)	3.10 ± 1.12[b]
Sebastes pinniger (Canary rock fish)	3.31 ± 0.37[b]
Sardinella aurita (Sardine)	3.47 ± 0.25[a]
Diplodus vulgaris (Two-banded seabream)	3.61 ± 0.09[c]
Boops (Bogue)*	3.64 ± 0.22[a]
***Micromesistius poutassou* (Blue whiting)**	**3.94 ± 1.40**
Oncorhynchus gorbusha (Pink salmon)	3.95 ± 0.79[b]
Symphodus cinereus (Grey wrasse)	4.15 ± 0.10[c]
Mallotus villosus (Capelin)	5.10 ± 0.89[b]
Boops (Bogue)*	5.62 ± 0.11[c]
Liza aurata (Golden grey mullet)	5.96 ± 0.13[c]
Sardinops sagax (Sardine)	6.43 ± 1.64[b]
***Capros aper* (Boarfish)**	**7.28 ± 3.22**
Mullus barbatus (Red mullet)	8.12 ± 0.27[c]
Clupea harengus pallasi (Pacific herring)	10.78 ± 0.68[b]
***Clupea harengus* (Herring)**	**11.20 ± 2.96**

[a] (Özogul and Özogul 2007); [b] (Huynh and Kitts 2009); [c] (Prato and Biandolino 2012)

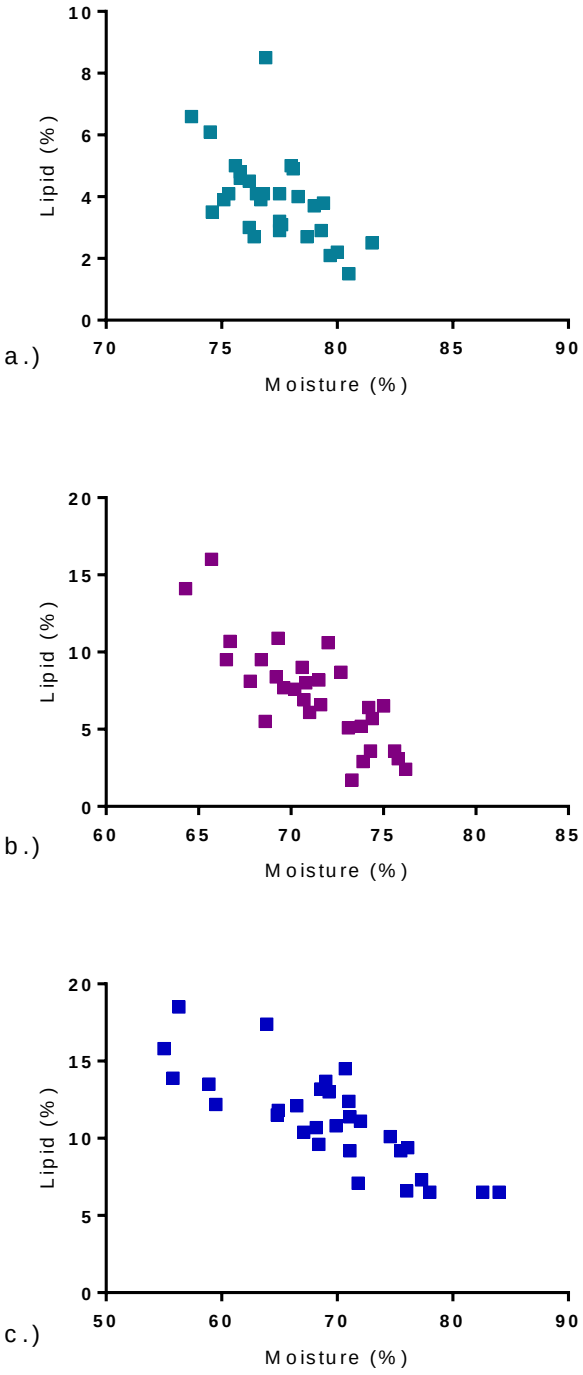

Figure 3.2. The observed correlation in whole body moisture (%) and lipid (%) content of a) blue whiting, b) boarfish, and c) herring (*n*=30).

3.2. Protein amino acid composition

The amino acid profile of the three species of fish was analysed and compared to the essential amino acid (EAA) requirements of human adults and salmon, as well as the composition of a range of common food protein sources as reported in previous publications (Table 3.2.). The amino acid profiles of the three fish were similar. For most EAA blue whiting had the lowest concentration. Herring had the highest concentration of the branched chain amino acids (BCAA); leucine, isoleucine and valine. These levels of BCAA were similar to that found in egg, soy and pea. However, milk and whey protein hydrolysate had higher levels of these EAA. Of the three fish species, boarfish had the optimum EAA ratio for human and salmon requirements. Cystine is a significantly limiting amino acid of these fish species in terms of human nutrition. However, they all contain high concentrations of methionine from which cystine can be synthesised endogenously.

Table 3.2. Recommended essential amino acid (EAA) daily intake per kg weight of an adult and recommended EAA composition ratio (mg g^{-1} protein) for adults and Atlantic salmon compared to the EAA composition ratio (mg/g protein) found in whole blue whiting (BW), boarfish (BF) and herring (HR) (pooled samples *n* = 6) as well as a range of common food protein sources; milk, whey protein hydrolysate (WPH), egg, soy and pea.*Data for salmon is an average of the requirements of all age classes.

	Amino acid requirements			BW	BF	HR	Milk[3]	WPH[4]	Egg[5]	Soy[6]	Pea[6]
	Adults[1]		Salmon*[2]								
Amino acid	mg kg^{-1} per day	mg g^{-1} protein	mg g^{-1} feed	mg g^{-1} protein							
Arginine	-	-	17	57	68	62	23	23	81	73	102
Histidine	10	15	7	27	24	44	23	13	25	26	25
Isoleucine	20	30	12	32	39	44	50	70	53	46	46
Leucine	39	59	21	57	69	80	81	102	86	79	73
Lysine	30	45	24	62	77	85	43	95	73	65	81
Methionine	10	16	-	35	40	44	23	25	30	13	10
Cystine	4	6	-	9	2	2	5	16	22	13	12
Methionine + Cystine	15	22	12	44	42	46	28	41	52	26	22
Phenylalanine + Tyrosine	25	30	25	51	63	48	91	-	94	82	78
Threonine	15	23	15	35	41	47	44	74	44	39	44
Tryptophan	4	6	3	-	-	-	23	-	13	13	10
Valine	26	39	17	42	46	56	59	58	68	49	51

[1] FAO/WHO/UNU (2002); [2] NRC (2011); [3] Medhammer *et al.* (2012); [4] Morato *et al.* (2013); [5] Gilbert *et al.* (2011); [6] Day (2013)

3.3. Lipid composition

Total lipids, lipid classes and fatty acid composition of triglycerides and phospholipids of boarfish, blue whiting and herring were analysed. The fish used for lipid composition analysis had averages of 4.7 ± 1.1%, 6.1 ± 1.0% and 10.8 ± 0.8% total lipids for BW, BF and HR, respectively (Table 3.3). Four lipid classes (TG, PL, GL and FFA) were obtained from the total lipids using solid phase extraction. The total lipids were principally composed of TG (91.2 – 95.7%; Figure 3.3). Herring had the highest amount of TG and this difference was found to be significant ($F(3, 24) = 11822$, $p < 0.001$) compared with BW and BF. Levels of PL and GL detected in the oil were similar for all three fish species. FFA were at 7% of total lipids in BW and BF, whereas in HR they were detected at a significantly lower concentration (2.8%; $F(3, 24) = 11822$, $p < 0.001$).

Triglycerides make up over 90% of the total lipids of these fish while phospholipids comprise a much smaller fraction. However, they are of growing interest in terms of nutritional value as well as having value in pharmaceutical and manufacturing applications (Burri *et al.* 2012). The fatty acid compositions of these two lipid classes were investigated using a GC-FID to analyse the derivatised FAME. Palmitic acid (C16:0) was the fatty acid found in the greatest concentration in all three fish species (averages of 20.1 – 23.6%). This was followed by oleic acid (C18:1n9) and docosahexaenoic acid (DHA; C22:6n3) at averages of 19.4 – 21.0% and 11.8 – 13.3%, respectively. The concentration of only one of the TG fatty acids varied significantly between the three fish. Myristic acid (C14:0) was found at a relative concentration of 10.6 and 9.6% in boarfish and herring, respectively, while blue whiting had a significantly lower concentration of 6.3% ($F(26, 162) = 98.10$, $p < 0.05$; Table S3.1.).

Of the fatty acids analysed, total saturated fatty acids (SFA) made up 35.6% and 37.7% concentration of the fatty acids detected in boarfish and herring, respectively. While blue whiting had a significantly lower concentration (31.3%; $F(7, 48) = 757.5$, $p = 0.001$). Mono-unsaturated fatty acids (MUFA) were highest in blue whiting (43.2%), significantly more so than boarfish (38.1%) or herring (38.9%, $F(7, 48) = 757.5$, $p = 0.001$). Poly-unsaturated fatty acids (PUFA) were found at between 23.5% and 27%, with boarfish having a significantly higher relative abundance compared to herring ($F(7, 48) = 757.5$, $p = 0.01$; Table 3.4.). No significant differences in the relative abundances of omega 3s, omega 6s, DHA or EPA were found between the three fish species. The sum of the omega-3 fatty acids ranged from 19.6%

in herring to 22.1% in blue whiting, while the omega-6 fatty acids were detected at much lower concentrations with total sums ranging from 2.3 – 2.9% (Table 3.4.). The omega-3 fatty acids DHA and eicosapentaenoic acid (EPA; C20:5n3) were at high relative abundances. DHA concentrations have been outlined above and EPA ranged from averages of 5.85 ± 1.24% in herring to 8.07 ± 1.31% in blue whiting. The ratio of omega-3 to omega-6 fatty acids was between 8.2 and 9.4, while the ratio of DHA to EPA was between 1.6 and 2.3. Blue whiting, boarfish and Atlantic herring, when compared to a range of other important marine fish, had low relative abundance of PUFA and omega-3 fatty acids. Atlantic herring from this study contained the lowest levels of PUFA and omega-3 fatty acids compared to 28 other fish. Blue whiting and boarfish were the 5th and 6th lowest for total PUFA and 7th and 9th for total omega-3 levels, respectively. However, looking specifically at the omega-3 fatty acids DHA and EPA, Atlantic herring had higher relative abundances compared to 20% of the other fish, while boarfish had higher relative abundances compared to one third of the fish. Interestingly, blue whiting had higher DHA levels compared to 25% of the fish but was in the top third of fish compared for EPA concentrations (Table 3.5.).

In contrast to the TG fatty acid composition, the PL fatty acid composition of the three fish had several significant variations (Table S3.2.). Palmitic acid, DHA, and oleic acid were also the three most abundant fatty acids detected in the PL fraction. However, DHA was at a slightly higher relative concentration in boarfish and herring and equal in blue whiting, compared to palmitic acid. Although, DHA was the most abundant fatty acid in all three fish it was detected at a significantly higher relative concentration in herring (35.38 ± 4.75%) compared to blue whiting (24.63 ± 2.19%) and boarfish (29.35 ± 2.24%, $F(26, 162) = 449.7$, $p < 0.001$). EPA was detected in herring and blue whiting at 10.26 ± 0.74% and 11.96 ± 0.64% concentration, respectively. The PL extracted from boarfish oil only had 5.76 ± 1.15% EPA, significantly less than the other two fish species ($F(26, 162) = 449.7$, $p < 0.001$). Significant differences in the relative concentration of palmitic acid, stearic acid (C18:0), oleic acid and α-linolenic acid (C18:3n3) were also found between the three fish.

PL levels of SFA were similar to the TG fraction, ranging from 30.3% to 36.3%. Whereas the relative abundances of the total MUFA and PUFA were the opposite. MUFA varied significantly from 14.9 ± 1.8% in herring to 27.3 ± 1.6% in blue whiting (ANOVA, $p < 0.001$). PUFA made up the largest fraction (42 – 50%). Herring contained the highest relative concentration of PUFA (49.9 ± 4.3%). The relative concentration of PUFA in herring was

significantly higher than that of blue whiting but not boarfish (42.5 ± 2.5% and 43.1 ± 1.6% respectively; $F(7, 48) = 219.4$, $p < 0.05$). The difference in PUFA relative concentration is the result of higher relative levels of omega-3 fatty acids in boarfish (Table 3.4.).

Table 3.3. Morphometrics (wet weight (g) and fork length (cm)) and total lipid content (%) of fish used for fatty acid composition analysis of blue whiting, boarfish and herring.

	Weight (g)			Length (cm)			Total lipids (%)		
	BW	BF	HR	BW	BF	HR	BW	BF	HR
Fish 1	202.9	56.1	108.2	31.0	12.0	20.4	4.5	5.0	11.8
Fish 2	120.2	51.6	87.8	24.8	12.0	19.0	6.2	5.9	10.4
Fish 3	185.8	56.8	86.6	30.5	11.7	18.6	3.5	7.4	10.0

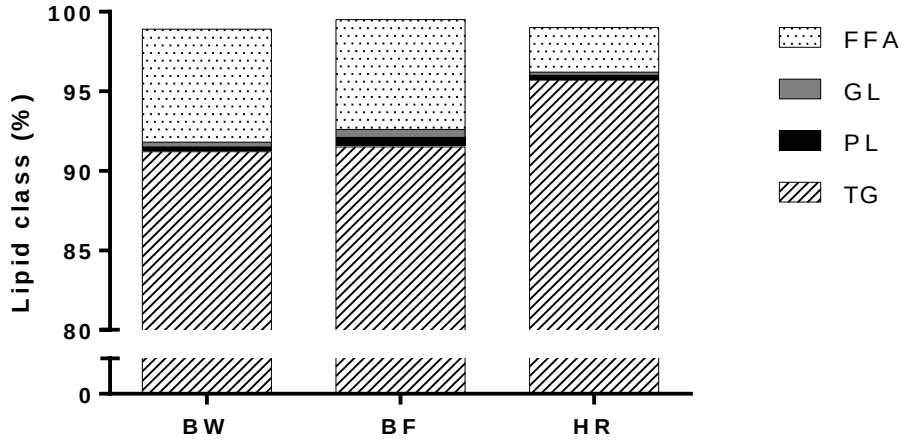

Figure 3.3. The percent proportion of four lipid classes (triglycerides, TG, phospholipids, PL, glycolipids, GL and free fatty acids, FFA) obtained from the total lipids of blue whiting (BW), boarfish (BF) and herring (HR) (*n*=3).

Table 3.4. The sums of triglyceride and phospholipid fatty acids, grouped by level of saturation and double bond location, detected in blue whiting, boarfish and herring (*n*=3).

	BW	BF	HR	Sig (*p* <)
Triglycerides				
SFA ∑	31.3 ± 0.9[a]	35.6 ± 3.1[b]	37.7 ± 0.4[b]	0.001
MUFA ∑	43.2 ± 1.5[a]	38.1 ± 2.0[b]	38.9 ± 1.5[b]	0.001
PUFA ∑	25.6 ± 2.3[ab]	27.0 ± 1.2[a]	23.5 ± 1.1[b]	0.05
PUFA/SFA	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.0	ns
Omega 3 ∑	21.6 ± 2.2	22.1 ± 1.3	19.6 ± 0.9	ns
Omega 6 ∑	2.3 ± 0.1	2.9 ± 0.7	2.4 ± 0.4	ns
Omega 3: Omega 6	9.4 ± 1.2	8.2 ± 2.6	8.2 ± 1.1	ns
DHA: EPA	1.6 ± 0.6	2.3 ± 0.7	2.2 ± 0.8	ns
Phospholipids				
SFA ∑	30.3 ± 3.1[a]	36.3 ± 1.1[b]	35.2 ± 2.9[b]	0.05
MUFA ∑	27.3 ± 1.6[a]	20.6 ± 0.7[b]	14.9 ± 1.8[c]	0.001
PUFA ∑	42.5 ± 2.5[a]	43.1 ± 1.6[ab]	49.9 ± 4.3[b]	0.05
PUFA/SFA	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.2	ns
Omega 3 ∑	38.0 ± 2.4[a]	36.9 ± 1.1[a]	46.3 ± 4.4[b]	0.001
Omega 6 ∑	1.7 ± 0.2	2.4 ± 0.1	1.1 ± 0.2	ns
Omega 3: Omega 6	22.7 ± 2.5[a]	15.7 ± 1.1[a]	43.0 ± 10.9[b]	0.001
DHA: EPA	2.1 ± 0.2	5.4 ± 1.3	3.5 ± 0.7	ns

Table 3.5. The sums of PUFA and omega-3 fatty acids and relative concentration of DHA and EPA from total lipids of important marine fish in the Mediterranean, Aegean and Black Seas and the Pacific Ocean as reported in three published research articles as well as the results from this study for blue whiting, Atlantic herring and boarfish (triglycerides fraction only).

	PUFA ∑	Omega 3 ∑	DHA	EPA
Herring	**23.5**	**19.6**	**11.8**	**5.9**
Pacific herring [b]	24.2	20.6	8.6	8.7
Capelin [b]	24.6	20.9	10.0	7.9
Mullet [a]	24.8	21.7	7.7	10.5
Blue whiting	**25.6**	**21.6**	**11.9**	**8.1**
Boarfish	**27.0**	**22.1**	**13.3**	**6.4**
Bogue [a]	27.5	26.0	18.7	5.1
Red mullet [c]	27.7	20.8	12.1	7.2
Grey wrasse [c]	28.8	21.1	9.9	6.8
Golden grey mullet [c]	28.9	21.5	10.8	6.1
Sardine [b]	31.0	27.6	13.3	11.7
Grass goby [c]	31.8	22.9	12.3	5.0
Pandora [a]	32.0	30.3	21.9	5.3
Picarel [c]	32.1	25.2	14.7	7.2
Salema [c]	32.1	26.8	17.4	7.2
Seabass [c]	32.5	23.1	13.8	6.9
Common sole [a]	33.6	31.1	18.7	7.7
C.T. Seabream [c]	34.0	27.0	17.2	7.5
Black goby [c]	34.2	23.4	12.5	6.1
Sand smelt [c]	34.7	26.1	15.7	8.6
Canary rock fish [b]	37.1	33.8	18.2	11.6
Pink Salmon [b]	38.9	35.0	19.3	8.2
Turbot [a]	41.1	38.4	30.3	5.3
Red scorpion fish [a]	41.6	37.0	28.0	4.7
Pacific hake [b]	43.5	38.4	22.1	12.9
Surf smelt [b]	44.5	38.8	20.7	12.9
Scad [a]	46.4	43.7	36.2	5.4
Walleye pollock [b]	47.6	44.9	29.0	12.4
Sardine [a]	56.8	51.2	32.7	11.4

[a] (Özogul and Özogul 2007); [b] (Huynh and Kitts 2009); [c] (Prato and Biandolino 2012)

3.4. Mineral composition

Homogenised samples of whole blue whiting, boarfish and herring were analysed for mineral (calcium, iron, magnesium, phosphorus, potassium, selenium and sodium; Table 3.6.) and heavy metal (arsenic, cadmium, copper, lead, mercury and zinc; Table 3.7.) content. Calcium was found in the greatest concentration. Blue whiting and boarfish had high levels of calcium; 1090 and 1520 mg $100g^{-1}$, respectively, while herring only contained 272 mg $100g^{-1}$. Phosphorus was the second most prevalent mineral and followed a similar trend as calcium; blue whiting and boarfish contained 622 and 789 mg $100g^{-1}$, respectively, while herring only contained 363 mg $100g^{-1}$. The calcium and phosphorus content in 100 g of blue whiting and boarfish is above the recommended daily intake for adults (Table 3.6). Sodium content of all fish was similar (~ 200-300 mg 100 g^{-1}) and within the recommended daily intake for adults (~ 200-500 mg $100g^{-1}$). All other minerals analysed were detected at levels significantly below the recommended daily intake for adults. In particular, selenium was detected at between only 0.04 and 0.06 mg $100g^{-1}$.

All heavy metals detected were below maximum allowable limits (MALs) specified by various regulatory bodies, except for the zinc levels found in boarfish. Zinc in boarfish was at 55.70 mg kg^{-1} and the MAL for zinc is at 30 mg kg^{-1} for fish (Nauen 1983). Boarfish also had the highest level of cadmium (0.124 mg kg^{-1} compared to 0.07 and <0.04 in blue whiting and herring, respectively). Arsenic, copper and mercury were at similar levels in all fish, although highest in blue whiting. Lead was detected at < 0.05 mg kg^{-1} in the three fish (Table 3.7.).

Table 3.6. The mineral content of whole blue whiting, boarfish and herring (mg $100g^{-1}$) and the recommended daily intake for adults (mg day^{-1}).

	mg $100g^{-1}$			mg day^{-1}	
	Blue whiting	Boarfish	Herring	male (19 - 65 yrs.)	female (19 - 50 yrs.)
Calcium	1090.00	1520.00	272.00	1000[a]	1000[a]
Iron	1.07	2.83	2.06	9 - 27[a]	20 - 59[a]
Magnesium	47.50	62.10	40.70	260[a]	220[a]
Phosphorus	622.00	789.00	363.00	700[b]	700[b]
Potassium	267.00	112.00	252.00	3510[c]	3510[c]
Selenium	0.06	0.05	0.04	34[a]	26[a]
Sodium	257.00	282.00	218.00	200-500[d]	200-500[d]
Zinc	1.29	5.57	1.33	4 - 14[a]	3 - 10[a]

[a] Joint FAO/WHO (1998)

[b] Institute of Medicine (1997)

[c] WHO (2012a)

[d] WHO (2012b)

Table 3.7. The heavy metal content (mg kg^{-1}) of whole blue whiting, boarfish and herring and the maximum allowable limit as specified by various regulatory bodies.

mg kg^{-1}	Blue whiting	Boarfish	Herring	MAL
Arsenic	2.44	1.34	1.62	3.00[e]
Cadmium	0.07	0.124	<0.04	1.00[e]
Copper	0.71	0.41	0.61	30.00[f]
Lead	<0.05	<0.05	<0.05	0.20[g]
Mercury	0.073	0.068	0.047	0.50[e]
Zinc	12.90	55.70	13.30	30.00[f]

[e] Bosch (2016)

[f] Nauen (1983)

[g] Joint FAO/WHO (1998)

4. DISCUSSION

This study aimed to provide a detailed report of the proximate composition of three LTL pelagic fish species; blue whiting, boarfish and herring. It focussed on these species caught in the Northeast Atlantic region, for which there are no such published data to-date.

These fish species were found to be high in protein (16% to 17%). This is a significant fraction of whole-body raw material when comparing it to other common protein sources. Whole milk contains approximately 3.2% protein, of which whey protein comprises only 20% (Medhammar *et al.* 2012). Eggs contain approximately 12% protein, while common plant protein sources such as soy and pea may contain 20 -40% protein (Day 2013; Marsh *et al.* 2013). When considering the efficacy of a raw material for protein powder production, total protein content, as well as the amino acid profile should be considered. There are 20 standard proteinogenic amino acids, of which nine are essential amino acids which the body cannot produce and therefore, need to be acquired in the diet. Protein synthesis is stimulated by EAA alone (Helms *et al.* 2014) and for dietary protein to be well incorporated into new protein it is necessary for the EAA to be at a ratio that resembles that of the protein to be synthesised, e.g. human or animal muscle (Gilbert *et al.* 2011). Furthermore, within the EAA, the BCAA (valine, leucine and isoleucine), and in particular leucine, stimulate protein synthesis (Kimball and Jefferson 2006). Egg was previously considered an excellent source for fulfilling EAA requirements and was traditionally used as the standard of comparison for measuring protein quality (Layman and Rodriguez 2009). Likewise, whey protein hydrolysate has become a popular raw material for protein powders marketed towards sports nutrition because of its high levels of BCAA, especially leucine. These foods are excellent sources of protein and BCAA. The amino acid ratio of WPH, however, is not balanced to the requirements of human nutrition. All essential amino acids are required together in adequate quantities and proper proportions for protein synthesis. A deficit in any one EAA limits protein synthesis (Srikantia 1981). Thus, the excess proportions of leucine in WPH cannot be fully utilised unless mixed with other proteins that provide higher levels of the limiting EAA in WPH.

Protein from pea and boarfish both have the closest matching EAA ratio to salmon requirements. However, salmon are naturally carnivorous and plant proteins can contain anti-nutritional factors that have been shown to negatively affect salmon health and growth (Francis *et al.* 2001; Bendiksen *et al.* 2011; Dalsgaard *et al.* 2012). Therefore, of the foods

compared here, boarfish protein would be considered the optimum protein source for use in salmon aquafeeds. In terms of human nutrition, of the foods compared here, milk had the closest matching EAA ratio to adult human requirements, followed closely by egg. Boarfish and blue whiting also had EAA ratios that were close to adult human requirements. Furthermore, there have been some studies reporting that fish proteins demonstrate higher satiating effects than other protein sources (Uhe *et al.* 1992; Borzoei *et al.* 2006; Pal and Ellis 2010). Thus far, intervention studies comparing the effects of different protein sources on body weight have proved inconclusive (Gilbert *et al.* 2011). However, this is an area that is becoming increasingly important, especially in Western society and further investigation using standardized techniques would benefit this area of research.

Considering the total protein content and the amino acid profiles of blue whiting, boarfish and herring, these LTL fish would be ideal raw material for protein powder production destined for human consumption or the aquaculture industry. To optimise a product destined for human nutrition protein derived from herring, and to a lesser extent blue whiting and boarfish, would benefit from mixing with other proteins (such as WPH) to create a product with an EAA ratio that more closely matches that recommended for humans.

Around one million tonnes of fish oil are produced every year (Ytrestøyl *et al.* 2011). Fish oils are generally considered to contain high levels of PUFA. PUFA have been associated with several different health benefits, the principal ones being improved cardio-vascular health, as well as healthy brain and eye function. The active metabolites in fish oils are primarily the omega-3 PUFA EPA and DHA, which are synthesised *de novo* in humans from the precursor essential fatty acid α-linolenic acid (ALA, 18:3n-3). However, it is an inefficient pathway and, EPA and DHA are therefore generally considered today as essential nutrients (Robertson *et al.* 2013). Marine fish build up high levels of PUFA, especially EPA and DHA via their food chains which begin with PUFA-synthesising algae (Lenihan-Geels *et al.* 2013). Fish oils are hence valuable nutrients, highly sought after as supplements for humans and animals.

Fish are classified as “fatty fish” if their lipid content is above 8% of their body weight and moderately fatty if it is between 4 – 8% (Tanakol *et al.* 1999). Therefore, from the results of this study, herring is considered a fatty fish, whereas blue whiting and boarfish are considered to be moderately fatty fish. Herring was the species studied here that had the highest levels of total lipids, as well as the highest proportion of TG, and lowest level of FFA. FFA are less stable than other oil fractions and are therefore more prone to oxidation and to

turning rancid. Thus, FFA levels are a key feature linked with the quality and commercial value of oils and fats (Mahesar *et al.* 2014). Higher quality oil will have a lower concentration of free fatty acids. Boarfish had the highest levels of polar lipids (phospholipids and glycolipids). Polar lipids are molecules with hydrophobic and hydrophilic ends. They are the major components of cell membranes in eukaryotic animal (phospholipids) and plant (glycolipids) cells. Their chemical structures make them useful natural surfactants that could be used by a range of industries (Holmberg 2001; Lourith and Kanlayavattanakul 2009). Furthermore, research had shown that polar lipids are more easily digested and absorbed and improve digestibility and absorption of triglycerides (Berger 2014). Therefore, they are becoming a valued fraction in fish oil supplements.

The fatty acid profile of the TG, the principal fraction of the fish oil, varied significantly between species, with herring containing the highest amount of SFA and lowest relative abundance of PUFA. Whereas blue whiting had significantly higher levels of MUFA and boarfish had significantly higher levels of PUFA. Comparing the PUFA and omega-3 content with other marine fish, Atlantic herring had low relative abundance of PUFA and omega-3 fatty acids. Boarfish and blue whiting, while having higher levels compared to Atlantic herring, still had lower levels compared to approximately three quarters of the fish. Considering total lipid content and fatty acid profile together, herring are a good source of fish oil. Herring has, and still is, used as a reduction species directed towards fishmeal and fish oil production (Neiland 2007). However, in recent years there has been a revival of a direct human consumption market (Cashion *et al.* 2017). In terms of maximising food security and sustainability, direct human consumption should be promoted when such an appetite exists but extraction of lipid fractions from waste streams may still provide valuable nutrients that could be used in a range of different products. Although still below average compared to the fish from other studies, boarfish and blue whiting contained moderate quantities of total lipids, PUFA and omega-3 fatty acids. The results from this study suggest that boarfish and blue whiting could be as good, if not better sources of fish oils, compared to herring.

PUFA made up a much larger part (nearly 50%) of the PL fraction from the three fish species. Furthermore, within this fraction herring had significantly higher levels of PUFA and omega-3 fatty acids compared to blue whiting and boarfish, respectively. In a number of clinical and pre-clinical trials, PL have been shown to be beneficial towards cardiovascular (Bunea *et al.* 2004; Fosshaug *et al.* 2011) and neurological health (Lee *et al.* 2010; Richter *et*

al. 2010) as well as treating symptoms of metabolic syndrome (Banni *et al.* 2011; Rossmeisl *et al.* 2012) and inflammatory-related diseases (Bjørndal *et al.* 2012; Grimstad *et al.* 2012; Vigerust *et al.* 2013), sometimes proving more successful than TG equivalents (Rossmeisl *et al.* 2012). PL are currently used for nutrition, cosmetics and drug delivery. The benefits of PL derive from their ability to form liposomes (Onyuksel and Rubinstein 2011; Hanin and Pepeu 2013). Most commercial PL are produced from plant sources (often soy) and from krill (marine copepods). Production of marine PL from fish or fish by-products is limited but there is potential for growth within this market (Burri *et al.* 2012).

Finally, fish are good sources of micronutrients, often superior to other animal-source foods in quality, quantity and bioavailability (Beveridge *et al.* 2013). They play an important role providing essential nutrients in poorer countries where diets are dominated by starchy staples (Beveridge *et al.* 2013; Béné *et al.* 2015). However, fish are also in intimate contact with their surrounding environment and have a tendency to bio-accumulate heavy metals in their tissues, thus providing a health risk to the consumer. In this study, we investigated both the mineral and heavy metal content of blue whiting, boarfish and herring.

Boarfish, followed by blue whiting and then herring, had the highest levels of total ash content. This result can be explained by the fact that boarfish have well armoured, bony bodies with long spines and thick scales whereas herring, and to a lesser extent blue whiting, are finely boned fish. The morphology of boarfish has caused challenges in the development of the fishery, whereby specialised equipment is required for catching and processing the fish. However, it also makes them an excellent raw material for mineral extraction. All of the minerals analysed, except for potassium and selenium, were found at the highest levels in boarfish. This is the first reports of mineral content in boarfish and it highlights this species as an excellent source of calcium, phosphorous and zinc. Comparing the mineral contents of blue whiting and herring detected in this study to other published studies (Martínez-Valverde *et al.* 2000; Tahvonen *et al.* 2000), herring had lower levels of all minerals. Whereas, the blue whiting analysed in this study had higher levels of potassium, zinc, sodium and calcium but lower levels of iron, magnesium and phosphorus.

Reviewing the heavy metal content of the three fish species, zinc in boarfish was the only heavy metal detected above specified MAL. The FAO previously set the MAL for zinc at 30 mg kg^{-1} for fish. However, in a more recent report they have set the upper level of zinc

intake for an adult man at 45 mg day^{-1} (FAO/WHO 1998). Since the likelihood of the average person consuming a kilogram of fish in a day is extremely low, it could be argued that the MAL for zinc in fish should be re-assessed. Arsenic is the only other heavy metal that was detected at levels close to the specified MAL. It would be important to closely monitor the content of this metal if these fish were to be processed for mineral streams.

This study provides an initial insight into the proximate composition of the LTL species blue whiting, boarfish and herring. It should be noted that nutrient content of fish is not stable. It varies in relation to many factors including species, sex, biological cycle, body part, season and place of development (Martínez-Valverde *et al.* 2000). This study focussed on fish in the Northeast Atlantic region. It has shown that three low value pelagic species contain significant amounts of high-quality proteins, lipids and minerals. Future studies would benefit from taking into account catch season, sex and even separating body parts in advance of analysis. Such work would build on this initial data and provide an even greater insight into the proximate composition ranges within these species.

The three species of fish studied here, and especially blue whiting and boarfish, are excellent sources of nutrients that have not previously been exploited to their full potential. The chemical composition of these fish, as outlined in this study, should help to direct future exploitation and guide appropriate product development which will in turn add significant value to these resources for human and animal nutritional applications.

Acknowledgements

This work was supported by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. [grant number EPSPG/2015/57]. This work was also supported in part by The APC Microbiome Institute, which is funded by Science Foundation Ireland (Grant Number SFI/12/RC/2273).

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SUPPLEMENTARY MATERIAL

Table S3.1. Triglyceride fatty acid composition (corrected FID response area relative percentages) of blue whiting, boarfish and herring (*n*=3).

	Blue whiting			Boarfish			Herring			Sig
	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	*p* <
SFA										
C10:0	0.01 ± 0.0	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	ns
C12:0	0.07 ± 0.01	0.05 ± 0.01	0.08 ± 0.04	0.10 ± 0.00	0.09 ± 0.00	0.06 ± 0.00	0.11 ± 0.01	0.16 ± 0.04	0.07 ± 0.00	ns
C14:0	5.90 ± 0.84	5.86 ± 1.15	7.17 ± 2.12	9.93 ± 1.69	16.42 ± 3.37	5.34 ± 0.36	9.99 ± 1.71	7.17 ± 1.12	11.77 ± 0.04	0.01
C16:0	21.81 ± 1.85	23.23 ± 1.91	20.63 ± 2.80	19.91 ± 1.50	17.22 ± 3.23	23.31 ± 0.81	23.09 ± 4.61	26.11 ± 1.59	21.52 ± 0.00	ns
C18:0	2.44 ± 0.0	3.27 ± 0.13	2.58 ± 0.01	3.85 ± 0.59	5.75 ± 1.12	3.73 ± 0.02	3.92 ± 0.49	3.57 ± 0.14	4.36 ± 0.03	ns
C20:0	0.18 ± 0.02	0.10 ± 0.08	0.18 ± 0.04	0.39 ± 0.04	0.33 ± 0.08	0.13 ± 0.10	0.25 ± 0.11	0.17 ± 0.03	0.40 ± 0.01	ns
C22:0	0.07 ± 0.00	0.06 ± 0.00	0.07 ± 0.00	0.09 ± 0.01	0.10 ± 0.02	0.06 ± 0.00	0.08 ± 0.03	0.06 ± 0.00	0.11 ± 0.00	ns
C24:0	-	-	-	0.05 ± 0.00	-	-	0.05 ± 0.00	-	-	ns
MUFA										
C12:1	-	-	0.02 ± 0.00	-	-	-	-	-	-	ns
C14:1	0.20 ± 0.01	0.15 ± 0.02	0.19 ± 0.06	0.12 ± 0.03	0.18 ± 0.06	0.09 ± 0.00	0.14 ± 0.03	0.10 ± 0.02	0.15 ± 0.03	ns
C16:1	8.08 ± 0.82	6.81 ± 0.73	6.87 ± 1.18	6.47 ± 1.82	0.67 ± 3.85	7.27 ± 0.31	6.88 ± 2.21	8.41 ± 0.69	5.05 ± 0.02	ns
C18:1n9	20.14 ± 0.58	24.58 ± 0.07	17.65 ± 0.39	16.48 ± 0.88	15.06 ± 0.87	26.81 ± 0.32	21.73 ± 4.34	26.52 ± 0.46	14.65 ± 0.06	ns
C18:1n11	4.80 ± 0.08	6.06 ± 0.10	3.75 ± 0.02	2.39 ± 0.10	2.09 ± 0.08	2.64 ± 0.06	2.44 ± 0.07	2.44 ± 0.07	1.92 ± 0.02	ns
C20:1	9.31 ± 0.96	7.18 ± 0.99	11.23 ± 1.57	9.94 ± 0.21	15.74 ± 0.19	3.78 ± 0.38	6.21 ± 3.62	2.88 ± 0.29	14.23 ± 0.24	ns
C22:1n9	0.37 ± 0.36	0.26 ± 0.26	1.72 ± 0.51	1.58 ± 0.51	2.37 ± 0.89	0.23 ± 0.22	1.23 ± 0.88	0.39 ± 0.04	0.94 ± 0.93	ns
C24:1	0.04 ± 0.01	0.04 ± 0.00	0.07 ± 0.00	0.09 ± 0.05	0.18 ± 0.09	0.04 ± 0.01	0.10 ± 0.04	0.06 ± 0.01	0.09 ± 0.00	ns

Table S3.1. Triglyceride fatty acid composition (corrected FID response area relative percentages) of blue whiting, boarfish and herring (*n*=3).

Continued.

	Blue whiting			Boarfish			Herring			Sig
	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	*p* <
PUFA										
C18:2n6	1.46 ± 0.04	1.60 ± 0.01	1.47 ± 0.04	1.45 ± 0.27	1.80 ± 0.42	1.37 ± 0.01	1.26 ± 0.08	1.37 ± 0.03	1.53 ± 0.00	ns
C18:3n6	0.15 ± 0.00	0.18 ± 0.0	0.16 ± 0.02	0.13 ± 0.02	0.11 ± 0.04	0.11 ± 0.00	0.10 ± 0.00	0.11 ± 0.00	0.15 ± 0.00	ns
C18:3n3	0.66 ± 0.03	1.19 ± 0.01	0.89 ± 0.20	0.78 ± 0.15	1.10 ± 0.22	0.68 ± 0.02	0.65 ± 0.02	0.68 ± 0.02	0.83 ± 0.01	ns
C20:2	0.42 ± 0.06	0.34 ± 0.07	0.18 ± 0.17	0.41 ± 0.01	0.65 ± 0.05	0.06 ± 0.01	0.32 ± 0.10	0.15 ± 0.07	0.47 ± 0.00	ns
C20:3n6	0.06 ± 0.01	0.07 ± 0.00	0.06 ± 0.00	0.15 ± 0.00	0.12 ± 0.00	0.10 ± 0.01	0.11 ± 0.04	0.08 ± 0.01	0.13 ± 0.02	ns
C20:4n6/C20:3n3	1.76 ± 0.10	1.44 ± 0.15	1.92 ± 0.16	1.85 ± 0.45	2.22 ± 0.74	1.54 ± 0.02	1.77 ± 0.50	1.39 ± 0.11	1.29 ± 0.00	ns
C20:5n3	7.35 ± 0.35	9.91 ± 0.84	6.94 ± 0.42	7.13 ± 2.09	3.97 ± 0.00	8.02 ± 0.05	5.89 ± 0.90	7.35 ± 0.57	4.32 ± 0.06	ns
C22:2	0.08 ± 0.00	0.01 ± 0.00	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.02	0.05 ± 0.02	0.06 ± 0.00	0.04 ± 0.00	0.01 ± 0.00	ns
C22:4	0.19 ± 0.04	0.15 ± 0.06	0.23 ± 0.07	1.42 ± 0.22	0.49 ± 0.20	0.25 ± 0.03	0.90 ± 0.77	0.17 ± 0.04	0.34 ± 0.01	ns
C22:5n3	0.92 ± 0.18	0.43 ± 0.10	0.91 ± 0.21	1.50 ± 0.55	2.61 ± 1.00	0.73 ± 0.07	1.28 ± 0.80	0.56 ± 0.08	1.74 ± 0.06	ns
C22:6n3	13.57 ± 2.86	7.10 ± 1.66	14.98 ± 4.03	13.72 ± 1.00	12.61 ± 1.17	13.58 ± 1.36	11.47 ± 2.94	10.06 ± 1.54	13.92 ± 0.46	ns

Table S3.2. Phospholipid fatty acid composition (corrected FID response area relative percentages) of blue whiting, boarfish and herring (*n*=3).

	Blue whiting			Boarfish			Herring			Sig
	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	*p* <
SFA										
C10:0	0.01 ± 0.00	0.01 ± 0.00	-	0.01 ± 0.00	-	0.01 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	ns
C12:0	0.03 ± 0.00	0.03 ± 0.02	0.68 ± 0.07	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	ns
C14:0	2.26 ± 0.02	1.32 ± 0.19	2.63 ± 0.20	1.98 ± 0.53	2.36 ± 0.06	1.87 ± 0.06	1.81 ± 0.44	1.73 ± 0.45	0.95 ± 0.05	ns
C16:0	26.03 ± 0.09	28.20 ± 2.27	20.35 ± 1.09	26.27 ± 4.41	25.75 ± 0.82	28.34 ± 0.48	32.73 ± 4.85	33.14 ± 3.99	27.66 ± 0.59	0.001
C18:0	2.56 ± 0.02	3.92 ± 0.10	2.46 ± 0.18	6.17 ± 0.21	8.76 ± 1.70	6.47 ± 0.07	2.29 ± 0.12	2.49 ± 0.09	2.42 ± 0.02	0.001
C20:0	0.04 ± 0.03	0.07 ± 0.00	0.06 ± 0.04	0.11 ± 0.02	0.23 ± 0.04	0.15 ± 0.00	0.08 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	ns
C22:0	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.00	0.12 ± 0.00	0.10 ± 0.02	0.12 ± 0.00	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	ns
C24:0	-	0.05 ± 0.00	-	0.15 ± 0.00	-	-				ns
MUFA										
C12:1	-	-	-	-	-	-	-	-	-	ns
C14:1	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	ns
C16:1	2.66 ± 0.02	1.73 ± 0.17	2.45 ± 0.01	2.13 ± 0.42	1.97 ± 0.40	2.17 ± 0.03	2.13 ± 0.38	2.66 ± 0.52	1.76 ± 0.05	ns
C18:1n9	17.25 ± 0.07	19.77 ± 0.68	20.95 ± 2.31	14.37 ± 1.12	14.37 ± 0.23	13.14 ± 0.02	8.10 ± 0.63	11.25 ± 1.13	9.15 ± 0.09	0.001
C18:1n11	2.87 ± 0.01	3.59 ± 0.10	2.78 ± 0.07	1.87 ± 0.11	1.71 ± 0.02	1.63 ± 0.00	2.58 ± 0.17	2.38 ± 0.25	1.92 ± 0.02	ns
C20:1	2.15 ± 0.02	1.95 ± 0.09	2.68 ± 0.10	1.36 ± 0.12	2.57 ± 0.86	2.50 ± 0.08	0.94 ± 0.04	0.88 ± 0.15	0.43 ± 0.01	ns
C22:1n9	2.82 ± 0.01	2.71 ± 0.02	2.77 ± 0.21	7.26 ± 0.16	1.74 ± 0.02	2.50 ± 0.82	2.30 ± 0.01	2.51 ± 0.04	2.46 ± 0.02	ns
C24:1	0.05 ± 0.00	-	0.05 ± 0.00	0.26 ± 0.05	0.17 ± .01	0.15 ± 0.00		0.05 ± 0.00	0.03 ± 0.00	ns

Table S3.2. Phospholipid fatty acid composition (corrected FID response area relative percentages) of blue whiting, boarfish and herring (*n*=3).

Continued.

	Blue whiting			Boarfish			Herring			Sig
	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	Fish 1	Fish 2	Fish 3	*p* <
PUFA										
C18:2n6	0.90 ± 0.00	0.96 ± 0.03	1.10 ± 0.05	1.08 ± 0.08	0.64 ± 0.09	0.89 ± 0.34	0.64 ± 0.05	0.55 ± 0.06	0.41 ± 0.00	ns
C18:3n6	0.08 ± 0.01	0.13 ± 0.01	0.11 ± 0.02	0.05 ± 0.01	0.12 ± 0.02	0.10 ± 0.00	0.07 ± 0.04	0.09 ± 0.01	0.05 ± 0.01	ns
C18:3n3	0.29 ± 0.01	0.40 ± 0.03	0.39 ± 0.00	0.14 ± 0.01	0.18 ± 0.08	0.19 ± 0.00	0.23 ± 0.02	0.25 ± 0.05	0.13 ± 0.00	0.05
C20:2	0.20 ± 0.00	0.16 ± 0.02	0.23 ± 0.00	0.21 ± 0.01	0.30 ± 0.00	0.20 ± 0.00	0.11 ± 0.02	0.10 ± 0.01	0.09 ± 0.01	ns
C20:3n6	0.06 ± 0.00	0.09 ± 0.02	0.16 ± 0.00	0.06 ± 0.01	0.12 ± 0.01	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.04	-	ns
C20:4n6/C20:3n3	0.26 ± 0.01	0.34 ± 0.04	0.24 ± 0.03	0.16 ± 0.03	0.74 ± 0.01	0.45 ± 0.16	0.09 ± 0.03	0.11 ± 0.01	0.08 ± 0.00	ns
C20:5n3	11.27 ± 0.02	11.79 ± 0.32	12.82 ± 1.99	7.33 ± 0.27	4.59 ± 0.41	5.37 ± 0.02	11.27 ± 0.21	9.98 ± 0.30	9.53 ± 0.17	0.001
C22:2	0.04 ± 0.00	0.08 ± 0.02	0.05 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.01				ns
C22:4	0.23 ± 0.01	0.20 ± 0.01	0.30 ± 0.01	0.86 ± 0.21	1.33 ± 0.30	0.91 ± 0.05	0.52 ± 0.18	0.24 ± 0.06	0.35 ± 0.01	ns
C22:5n3	0.97 ± 0.01	0.72 ± 0.08	1.54 ± 0.44	1.88 ± 0.40	1.51 ± 0.11	1.50 ± 0.01	0.50 ± 0.08	0.45 ± 0.05	0.52 ± 0.01	ns
C22:6n3	26.98 ± 0.29	21.71 ± 3.00	25.20 ± 2.63	26.20 ± 5.56	30.66 ± 1.06	31.19 ± 1.34	33.25 ± 5.95	30.94 ± 6.15	41.96 ± 0.60	0.001

Chapter 4

Bacteriomes and viromes of three marine pelagic fish are species specific

ABSTRACT

Viruses are the most abundant biological entities on Earth. In recent decades, the importance of the ecological link between bacterial viruses (bacteriophages) and their hosts has been recognized and it is now appreciated that the roles of viruses must be understood to fully comprehend microbial physiology, population dynamics and evolution. Ocean ecosystems harbour an abundant and diverse population of microbiota and fish are important members of these habitats that can act as microbial reservoirs. In this study, metagenomics has been used to characterize the bacteriome and virome of three commercially-important Northeast Atlantic pelagic fish species; blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*) and herring (*Clupea harengus*). The results reveal that fish are, in themselves, reservoirs of extensive bacterial and viral genetic diversity. The bacterial microbiota of these fish were dominated by the phylum Proteobacteria, followed by Tenericutes and Spirochaetes. Seven phyla were only recorded in one fish species and only two bacterial families (*Vibrionaceae* and *Lactobacillaceae*) were detected in the core microbiota of all three fish. These fish also have unique and novel viromes that separate distinctly in relation to species, especially in the case of blue whiting. *Microviridae* was the most abundant viral family followed by the *Caudovirales* families, *Myoviridae*, *Podoviridae* and *Siphoviridae*. Clusters of fish-associated *Microviridae* and *Circoviridae* sequences, distinct from anything characterised in the NCBI database, are reported. Comparison with ocean and human virome sequences showed fish viromes separating and forming distinct ecological communities.

1. INTRODUCTION

The pelagic oceans make up the largest environment on Earth (Angel 1993). Although this vast area likely holds significant biodiversity, our knowledge of it is inadequate and far behind many more easily accessible regions (Webb *et al.* 2010). This is especially worrying because of the vital ecosystem services that are provided by this ecosystem, which could be in jeopardy due to the current risks of climate change and biodiversity loss (Game *et al.* 2009; Robison 2009).

Microbes, in particular viruses, and most specifically bacteriophage, are the most abundant biological entities on Earth (Prigent *et al.* 2005; Prestel *et al.* 2013). Viruses have been found in every habitat where life has been observed and infect all life forms, from multicellular eukaryotes to single cell bacteria. Viruses that infect bacteria are known as bacteriophages (or phages), and they influence the bacterial community composition of habitats (Zhang *et al.* 2011).

In many habitats, viruses outnumber cells by as much as 10:1 (Ogilvie and Jones 2015). The oceans are considered the largest reservoir of viruses, with an estimated 10^7 particles per millilitre of seawater, equating to a total sum of 10^{30} viruses (Suttle 2007; Alavandi and Poornima 2012; Parikka *et al.* 2017). Marine phages control the microbial communities in the oceans, with reports that up to 70% of marine bacteria may be infected by phages, which kill approximately 20% of the microbial biomass per day (Suttle 2007). It is recognised that these dynamic microbial communities play an important role in the ecosystem services that the oceans provide. However, a complete understanding of the specific functions will remain unknown until the taxonomic composition and genetic functioning of these communities is determined (Coutinho *et al.* 2018).

The bacteriome and virome are the combined genetic material of bacteria and viruses found in a particular environment, respectively. The virome is the section of the microbiome that contains the greatest genetic diversity, and the extent of its global influence is only beginning to be understood (Suttle 2005; Zhang and Gui 2018). However, large-scale studies such as *The Marine Viromes of Four Oceanic Regions* (Angly *et al.* 2006), *The Pacific Ocean Virome* (Hurwitz and Sullivan 2013), *The Tara Oceans Expedition* (Brum *et al.* 2015; Sunagawa *et al.* 2015) and *Decoupling Function and Taxonomy in the Global Ocean Microbiome* (Louca

et al. 2016) have begun to uncover the magnitude and diversity of the marine "viral dark matter" and other marine microbial genomes.

While microbial ecologists are beginning to build a picture of the free-living microbiome in the pelagic oceans, knowledge of the microbial communities associated with the higher organisms of these habitats is sparse. Gut associated microbiotas are remarkably different from other free-living microbiotas from across the biosphere (Ley *et al.* 2008). However, fish and other marine animals have a unique and intimate interaction with their surrounding environment and, in turn, with the associated microbiome (Egerton *et al.* 2018). In this way, they act as reservoirs of unique microbial genetic diversity available to potentially influence external microbial communities.

Minimal research has focussed on teleost microbiota and metagenomic studies relating to fish are still relatively few (Hayes *et al.* 2017). The pelagic oceans supply > 80% of fish consumed globally (Pauly *et al.* 2002) and, aside from fisheries, these fish also provide nutrient, carbon and energy transport services (Holmlund and Hammer 1999). These characteristics make them an important topic for investigation.

The aim of this study was to characterize the gut microbiota of three commercially-important Northeast Atlantic pelagic fish species; blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*) and herring (*Clupea harengus*) sampled from the Celtic Sea and Atlantic Ocean region around Ireland, where they share the pelagic habitat. The results provide an initial insight into the bacterial and viral community compositions found in the guts of these species and afford a starting point from which teleost virome research can expand.

2. MATERIALS AND METHODS

2.1. Fish sampling

Sampling for all fish species occurred on-board the RV Celtic Explorer during the Western European Shelf Pelagic Acoustic Survey (WESPAS) between 06 June and 29 June 2017 (O'Donnell *et al.* 2017). Blue whiting sampled weighed 117 ± 33 g and had a fork length of 25.5 ± 1.5 cm, herring were 163 ± 25 g with a fork length of 26.6 ± 0.9 cm, while sampled boarfish weighed 75 ± 10 g and had a fork length of 15.7 ± 0.7 cm. Samples were collected at haul sites across the southern survey area (Table 4.1.). Longitude and latitude of fishing haul sites were mapped using ArcMap 10.5, coupled with Microsoft PowerPoint 2016 and Fireworks 4 (Figure 4.1.). For further information on survey details see the WESPAS 2017 Survey Report (O'Donnell *et al.* 2017).

2.2. Sample collection

Directly after capture and sorting of the haul, the peritoneal cavity of the sampled fish was opened, and adopting aseptic procedures, the intestine was freed from the connective tissues. The intestinal portion just after the pyloric caeca as far as the anus was separated from the rest of the gastrointestinal tract (GIT) and the intestinal contents were carefully squeezed out of the intestine from the proximal to distal colon, using sterile forceps. Samples were collected using either OMNIgene® GUT sample collection kits (DNA Genotek Inc., Ottawa, Canada), according to the manufacturer's protocols (16S microbiota samples) or placed directly into a sterile tube containing RNAlater, which was kept at 4^{o}C for 24 hours before being frozen at -80^{o}C (virome samples).

Table 4.1. Number of fishes sampled at different haul sites, species collected, and sequencing type carried out on each sample.

Haul #	**16S microbiota**			**Virome**		
	Blue whiting	Boarfish	Herring	Blue whiting	Boarfish	Herring
1		1				
2		1			3	
3	1					
4		1			3	
5		1	1		1	
6		1				
7	2			3		
8		1			2	
12	1			3		
13			4			7
14	2					
17	1					
19	1			4		

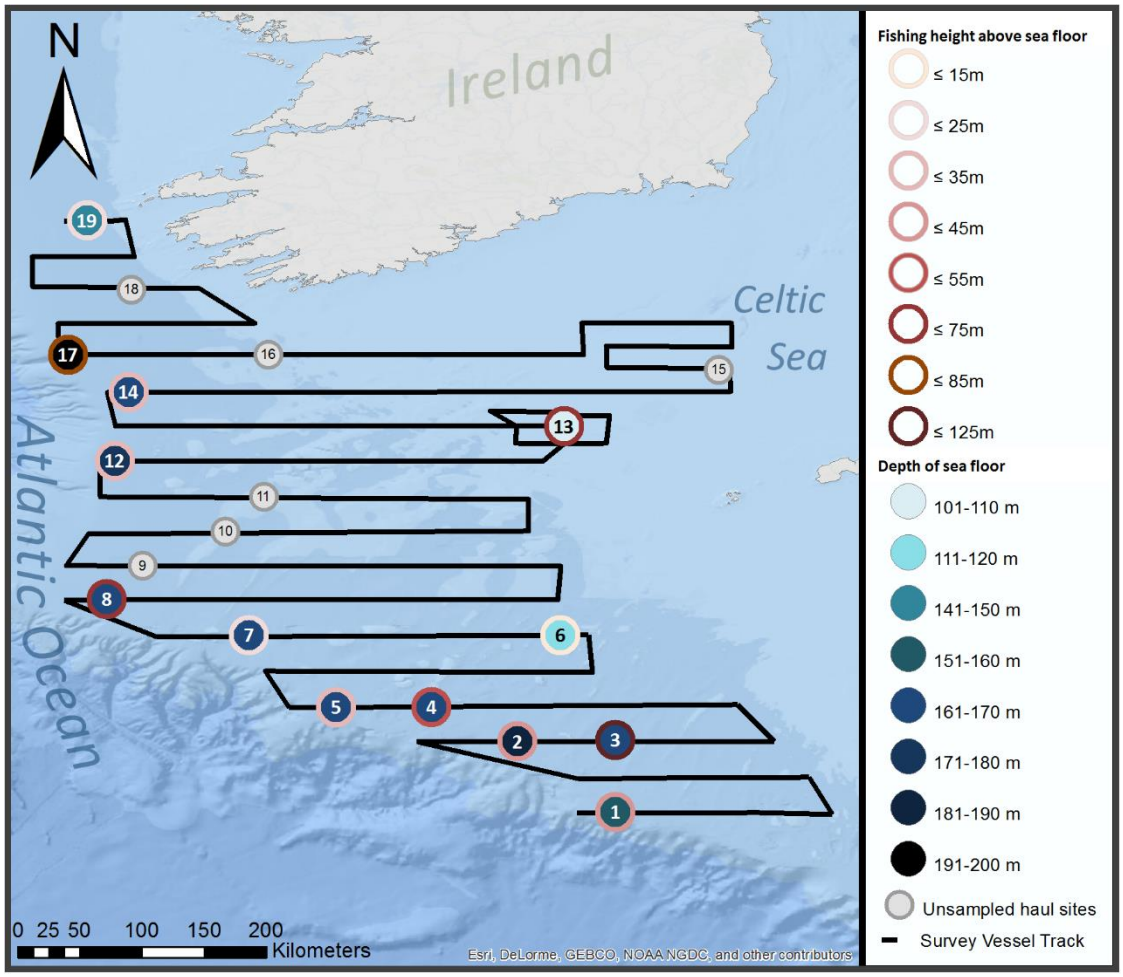

Figure 4.1. Survey direction and geographical locations of the fish haul sites at which samples were collected. Sea floor depth (blue) and fishing height above sea floor (red) at each haul site are represented using colour crescendos of the outer ring and central colour of the markers, respectively. This map has been adapted from an original map in the WESPAS 2017 survey report (O'Donnell *et al.* 2017).

2.3. DNA extraction and sequencing

2.3.1. 16S microbiota DNA

DNA extraction from samples was performed using a QIAGEN QIAamp PowerFecal DNA Isolation Kit (Qiagen Ltd, Manchester, England) and following the OMNIgene® GUT microbial DNA purification protocol. The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S rRNA metagenomic sequencing library protocol (Murphy *et al.* 2017; Watkins *et al.* 2017).The 16S V3-V4 rRNA gene primers also incorporate the Illumina overhang adaptors (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). The PCR reactions were performed in a 25 µL reaction volume containing DNA template, 12.5 µL Kapa HiFi HotStart PCR ReadyMix (Roche, Dublin, Ireland), 5 µL each of forward and reverse primers (1 µM), and PCR grade water to final volume. PCR amplification conditions included initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 5 min. PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland) before a second PCR reaction was completed to index each of the samples. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Indexing PCR reactions were performed in a 50 µL reaction volume containing 5 µL purified DNA, 5µL each of index 1 primer (N7xx) and index 2 primer (S5xx), 25µL 2x Kapa HiFi Hot Start Ready mix, 10µL PCR grade water. PCRs were completed as described above, but only eight amplification cycles were completed instead of 35. After quantifying the samples, using an Invitrogen Qubit 4 Fluorometer and a high sensitivity DNA quantification assay kit (BioSciences, Dublin, Ireland), they were pooled in an equimolar fashion. The sample pool was prepared following Illumina guidelines. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. Samples were sequenced on the MiSeq sequencing platform at the Teagasc Sequencing Facility, using a 2 x 300bp cycle kit, following standard Illumina sequencing protocols.

2.3.2. Viral DNA

To begin, intestinal samples, and the RNAlater they were stored in, were suspended in 10 mL of SM buffer (50 mM Tris-HCl; 100 mM NaCl; 8.5 mM $MgSO_4$; pH 7.5) and homogenised by vortexing for 5 min, before centrifuging twice (4 500 *g*, 10 min, 4 °C) to remove large particulates and bacterial cells. The resulting suspensions were filtered twice through a 0.45 µm pore diameter filter. Subsequently, NaCl (final conc. 0.5 M) and 10% (w/v) polyethylene glycol (PEG; average molecular weight 8 000) were dissolved in the samples and then chilled on ice for three hours. Viruses were then separated from solution using a centrifuge (4 500 *g*, 20 min, 4 °C). The viral-PEG pellet was suspended in 400 µL of SM buffer; viruses were then separated from the PEG by treating the samples with an equal volume of chloroform, vortexing and centrifuging. Clarified viral preparations were treated with 40 µL of 10 × Nuclease Buffer (50 mM $CaCl_2$; 10 mM $MgCl_2$), 20 U of DNase I, 10 U of RNase I (final concentrations; Ambion Inc., USA). Nucleases were inactivated at 70 °C for 10 min before samples were treated with 20 µL of 10% SDS and 2 µL of 20 mg L^{-1} proteinase K for 20 min at 56 °C. Remaining intact viruses were lysed by the addition of 100 µL of Phage Lysis Buffer (4.5 M guanidine thiocyanate; 45 mM sodium citrate; 250 mM sodium lauroyl sarcosinate; 562.5 mM β-mercaptoethanol; pH 7.0) and incubation at 65 °C for 10 min. Viral DNA was purified by two treatments with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and passing the resulting purified DNA through a QIAGEN Blood and Tissue Purification Kit and eluting samples in 50 µL of TE Buffer.

Viral DNA concentrations were equalised before amplification for sequencing using an Illustra GenomiPhi V2 kit (GE Healthcare, Little Chalfont, UK). Amplifications of purified viral DNA were performed on all samples as described by the manufacturer. Subsequently, any remaining inhibiting products were removed by passing the samples through the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Ireland Ltd., Arklow, Ireland). Finally, samples were prepared for sequencing using the Nextera XT library preparation kit (Illumina, San Diego, CA, USA) as described by the manufacturer. After quantifying the samples, using an Invitrogen Qubit 4 Fluorometer and high sensitivity DNA quantification assay kit (BioSciences, Dublin, Ireland), they were pooled in an equimolar fashion. The sample pool was prepared following Illumina guidelines. Samples were sequenced on the HiSeq sequencing platform (GATC Biotech AG

Ltd., Konstanz, Germany), using a 2 x 300bp cycle kit, following standard Illumina sequencing protocols.

2.4. Bioinformatics and statistical analysis

2.4.1. 16S rRNA sequencing analysis

Three hundred base pair paired-end reads were assembled using FLASH. Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME. De-noising, chimera detection and operational taxonomic unit (OTU) grouping at 98% similarity were performed using USEARCH v7 (64-bit) (Edgar 2010). OTU sequences were aligned using PyNAST and taxonomy was assigned using BLAST against the SILVA SSURef database release 123 (Quast *et al.* 2012). The α- and β-diversities were calculated using QIIME on weighted Unifrac distance matrices. The 16S rRNA OTU tables generated using QIIME were imported into R. Two dimensional NMDS ordination plots of 16S rRNA OTU taxa detected were performed using the R package 'phyloseq' (McMurdie and Holmes 2013) using Bray-Curtis calculated distances by the R package 'vegan' (Oksanen *et al.* 2013). A summary of the most abundant phylum, family or genus level taxa is provided, with only taxa that had an average relative abundance of ≥ 1% averaged across all samples displayed.

2.4.2. Virome sequencing analysis

Paired-end virome sequencing reads were downloaded from GATC, with an average of 7 266 878 (min. 3 298 988, max. 10 716 832) unprocessed reads per sample file. Adapters were removed, and the reads were trimmed to a Phred quality score of 30 across a 4bp sliding window using Trimmomatic version 0.32 (Bolger *et al.* 2014). Sequencing reads less than 70bp after quality trimming were discarded, resulting in an average of 2 481 766 (min. 971 545, max. 4 228 119) paired and 1 424 711 (min. 31 610, max. 4 082 498) unpaired high-quality reads per file. Viral contig assembly was performed on each sample individually using paired and unpaired reads with metaSPAdes version 3.10.0 (Nurk *et al.* 2017).

De novo phage detection was performed after assembly using the following criteria. Contigs ≥ 1 kb were selected, and their proteins predicted using Prodigal version 2.6.3 using

the 'meta' option, with Shine-Dalgarno training bypassed and a full motif scan performed on each individual contig (Hyatt *et al.* 2010). Proteins were queried locally using HMMER hmmscan version 3.1b1 against the prokaryotic viral orthologous groups (pVOGs) database (Finn *et al.* 2011; Grazziotin *et al.* 2016). A loose phage taxonomic classification was assigned to all assembled contigs using an in-house program, 'DemoVir' (unpublished), which utilises a voting system to assign taxonomy to phage contigs based on the similarity of encoded proteins to characterised phage proteins. To limit bacterial contamination making it into the selection of *de novo* predicted phage contigs, a strict cut-off of only contigs ≥ 5 kb that contained ≥ 3 pVOG hits per 10 kb were included into a final database of fish-associated phages. While for contigs assigned a putative *Microviridae* taxonomy, contigs ≥ 1 kb with ≥ 3 pVOG hits per 10 kb were retained in the final analysis. A total of 289 *Caudovirales* and 247 *Microviridae de novo* assembled phages were included in a final database, with only 2 putative phage contigs not assigned a top-level taxonomic rank.

2.4.3. Viral abundance

Strict criteria for the *de novo* detection of phages in faeces were implemented. The *de novo* detection of phage contigs was initiated through the detection of contigs with hits against the pVOG database. Therefore, only phages with similarity to known phage proteins were detected and analysed in this study, with additional phage diversity potentially not identified.

Family level putative taxonomy of *de novo* predicted phage contigs were assigned. High-quality sequencing reads from each fish faecal virome sample were mapped onto all phage contigs using Bowtie2 version 2.1.0 (Langmead and Salzberg 2012), in end-to-end mode. The average number of reads aligning to *de novo* predicted phage contigs per sample was 6.43% (min. 0.12%, max. 20.07%). The count matrix of reads aligned per phage contig was generated using SAMtools version 0.1.19 (Li *et al.* 2009). Subsequently, two dimensional NMDS ordination of the read count matrix for fish faecal phages was performed using Bray-Curtis distances, with groupings displayed for both fish species and haul site. Statistical variance analysis of reads mapping to *de novo* predicted phage contigs was performed in R using the Adonis function of the vegan package. Reads were also aligned to a reference viral database (RVDB, clustered database version 14.0) which includes known eukaryotic and prokaryotic viruses (Goodacre *et al.* 2018). The relative abundance (log scaled) of reads per

sample aligning to the top 20 most abundant viruses of the RVDB database is presented. A count matrix of reads aligned to all RVDB viruses, with at least ≥ 1 read mapping to a virus in one sample, was generated as described above. The average alignment rate of reads to the RVDB database was 5.14% (min. 1.28%, max. 15.26%) reads per sample. The two-dimensional ordination of reads aligning to RVDB reference viruses, highlighting fish species and fishing haul sites, was performed as described above.

A heatmap representation of the number of fish faecal-virome reads aligned to all putative *de novo* identified phage contigs was performed by calculating the relative abundance of reads aligned to all phage contigs per fish, followed by scaling the relative abundance across all fish. The heatmap is clustered by rows.

2.4.4. Viral phylogeny

The large terminase subunit amino acid sequences of *Caudovirales* phages were detected as follows. The full set of large terminase sequences from Pfam (PF04466) were downloaded and aligned using Muscle version 3.8.31 (Edgar 2004). The alignment was converted into a Hidden Markov Model (HMM) profile and queried against the protein sequences predicted in fish faecal-virome contigs. Only hits to the large terminase subunit HMM profile scan with e-values less than 1^{e-10} were considered significant. Following the detection of large terminase subunit proteins, amino acid sequences were extracted and aligned using Muscle.

The optimal amino acid substitution model, Blosum62, was computed with the 'phangorn' package in R (Schliep 2010). A maximum likelihood phylogenetic tree, bootstrapped 50 times, was visualized in R using the 'ggtree' package after importing the tree with 'treeio' (Yu *et al.* 2017; Yu *et al.* 2019).

The phylogeny of the capsid protein of *Microviridae* phages and replication protein of *Circoviridae* viruses was performed as described above for *Caudovirales* terminase sequences, using the *Microviridae* and *Circoviridae* amino acid sequences from Pfam PF02305 and PF02407, respectively, to initiate the search. However, only *Microviridae* capsid sequences ≥ 350 amino acids in length were analysed. In addition, only *Circoviridae* circular contigs were queried, with detected replication proteins ≥ 200 amino acids in length analysed. For the *Microviridae* capsid phylogeny, a random selection of NCBI *Microviridae* capsid protein sequences (*n* = 31) were included which were assigned to a subfamily taxonomic rank.

Similarly, random *Circoviridae* replication protein sequences from NCBI (*n* = 25) representing the two described genera were included in the analysis. The optimal amino acid substitution model for the *Microviridae* capsid protein and *Circoviridae* replication protein phylogenies was determined as the 'WAG' and 'Blosum62', respectively.

Following the phylogenetic analysis of *Caudovirales* large terminase subunits and *Microviridae* capsid proteins, additional hits were included from the '*Tara Ocean Viromes*' study (Brum *et al.* 2015; ENA study: PRJEB19352), the '*North Sea goes Viral*' study (Garin-Fernandez *et al.* 2018; ENA study: PRJEB21210) and the '*Healthy Human Gut Phageome*' study (Manrique *et al.* 2016; NCBI BioProject: PRJNA308867) following the same read processing, assembly, phage and protein detection protocols as outlined above. However, with exception, contigs were downloaded pre-assembled from the Tara Ocean virome study.

2.4.5. Statistical analysis

For bacteria 16S rRNA analysis, IBM SPSS Statistics 24 statistical software package was used. Statistical significance in α-diversity measures was assessed for Chao1 and Shannon indices. One-way ANOVA tests followed by Tukey post-hoc tests were used to determine the statistical significance of the 16S diversity measures. The Shannon diversity of viruses present in the faeces of each fish was calculated using the 'vegan' package in R (Goodacre *et al.* 2018). Non-parametric Kruskal-Wallis tests were used to determine statistical significance in the relative abundance of 16S rRNA microbial taxa between the three fish species. Statistical significance was accepted at $p < 0.05$.

3. RESULTS

3.1. Fish bacteriome

3.1.1. Fish 16S rRNA microbial diversity

After non-joining and chimera sequences were removed, sequencing yielded a total of between 121 698 and 285 647 sequence reads and between 136 and 526 OTUs per sample. Sequence coverage was ≥ 98% in all cases. Significant differences in α-diversity were found between the three fish species (Figure 4.2a., b.). Species richness in the gut communities was calculated by the Chao1 diversity index. Samples from boarfish were found to have a significantly higher 16S rRNA species richness compared to blue whiting and herring ($F(2, 16) = 21$, $p < 0.001$; Figure 4.2a.). Using the Shannon index, samples from blue whiting showed significantly higher diversity in comparison with boarfish samples ($F(2, 16) = 4.8$, $p < 0.05$; Figure 4.2b.). Furthermore, there were notable differences in the inter-sample variation in the three groups, with herring samples showing a large range in diversity levels (Figure 4.2b.). β-diversity analysis showed significant separation of the bacteriomes by fish species on the principle coordinate plots (Figure 4.3a., c., e.; PERMANOVA test, $R^2 = 0.44$ at genus level; $p < 0.001$).

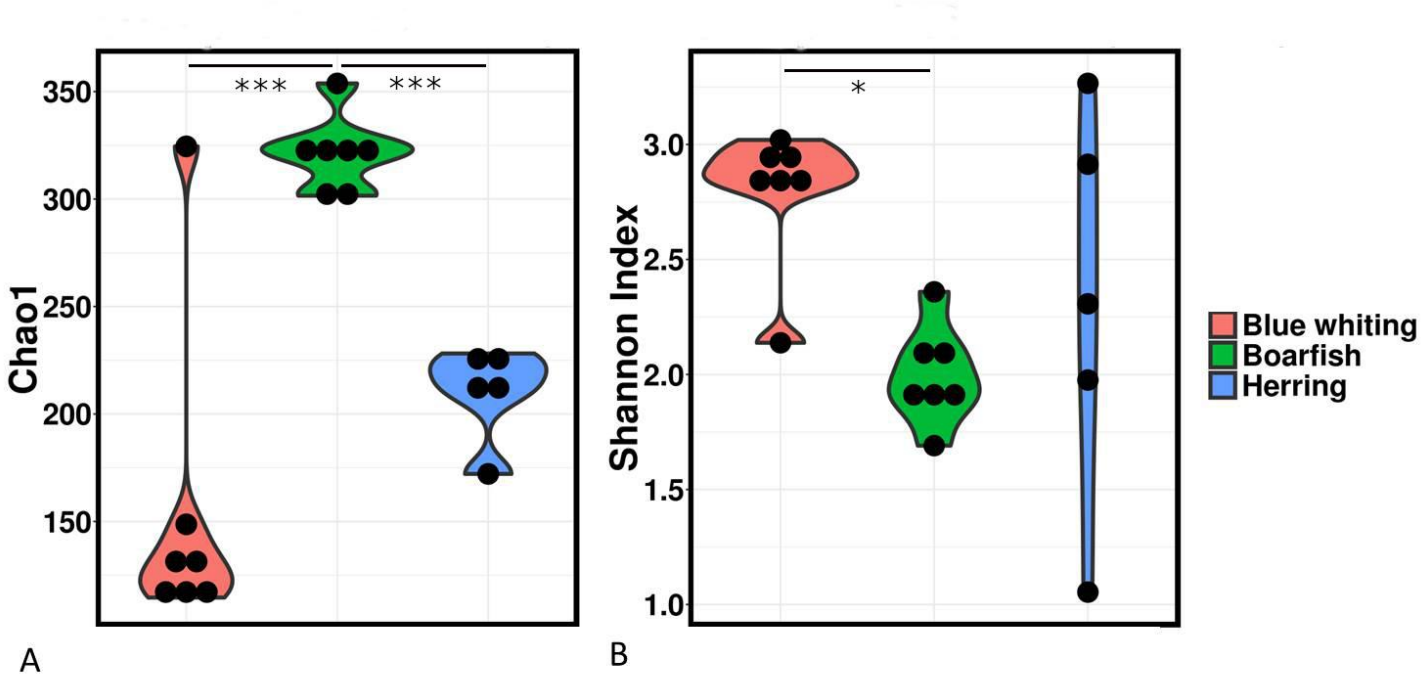

Figure. 4.2. The Chao1 (a.), and Shannon (b.) α-diversity measures of 16S rRNA microbiota gut community compositions in faecal microbiomes of three species of fish.

3.1.2. Fish 16S rRNA microbial composition

At the phylum, family and genus levels, the bacterial compositions of fish faecal microbiota are distinct between species (Figure 4.3a. – f.). Eighteen bacterial and two archaeal phyla were identified from the gut content of all fish species. Of these, seven phyla were only recorded in one fish species. Considering the three fish species together, Proteobacteria was the dominant phylum; ranging from 77 – 92%, 15 – 44% and 5 – 62% of OTUs in boarfish, blue whiting and herring, respectively. The remaining OTUs in the boarfish samples were mostly Firmicutes (1 – 7%) or unidentified blast hits (6 – 17%). Blue whiting samples were dominated by Spirochaetes (23 – 41%) while Bacteroidetes, Deferribacteres, Firmicutes and Tenericutes also made up a notable proportion. The herring samples had the highest inter-sample diversity. Considering the herring samples together, Tenericutes was the phylum that contained the second greatest number of OTUs after Proteobacteria. However, this result is skewed by a single sample where 81% of OTUs were identified as Tenericutes. Omitting this sample, Cyanobacteria was the second major phylum identified (Figure 4.3a., b.).

Analysis of the 16S rRNA compositional data at family and genus levels considered the core microbiota of each species (only OTUs present in every sample at a relative abundance of ≥ 0.01%; Astudillo-García *et al.* 2017). Considering the results at a family level, *Vibrionaceae* was the most dominant in all three species. Aside from this member, the 16S rRNA microbial composition at family level varied significantly between the three fish species (Figure 4.3c., d.). Boarfish (76.22 ± 15.60%) had a significantly higher number of OTUs in the *Vibrionaceae* family compared to blue whiting (29.68 ± 23.51%) and herring (28.15 ± 23.20%; $H(2) = 9.806$, $p < 0.01$). The core microbiota of boarfish consisted of only four families; *Vibrionaceae*, *Lactobacillaceae*, *Hahellaceae* and *Planctomycetaceae*. The families *Hahellaceae* and *Planctomycetaceae* were also detected in the core microbiota of herring, though not blue whiting. Blue whiting and herring's core microbiota were made up of nine and 17 families, respectively. *Mycoplamataceae* was the second most dominant family in herring (24.57 ± 30.75%). After *Vibrionaceae*, the most prevalent families in blue whiting were *Rikenellaceae* (14.73 ± 7.01%), *Brevinemataceae* (16.40 ± 8.00%), and *Brachyspiraceae* (12.34 ± 5.68%). These three families were found in the herring core microbiota as well, though in significantly lower abundance ($U(1) = 4$, $p < 0.05$, Supplementary Table S4.1.).

At genus level, herring had the most diverse core microbiota, followed by blue whiting and then boarfish (Figure 4.3e., f.). The only genera present in the core microbiota of all three species were the *Vibrionaceae* genera; *Enterovibrio, Protobacterium,* and *Vibrio* (Supplementary Table S4.2.).

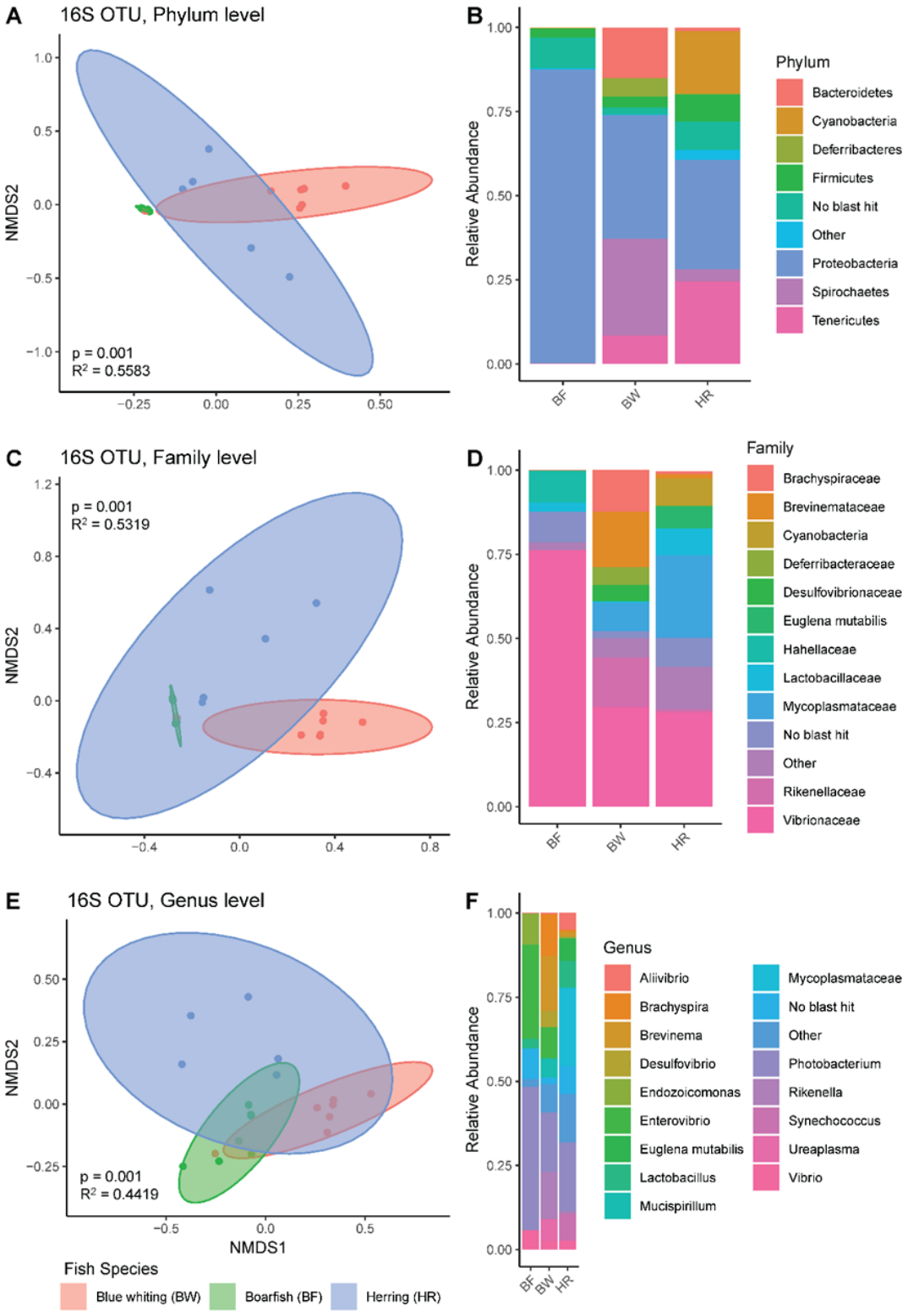

Figure 4.3. Bacterial 16S rRNA sequencing analysis. NMDS ordination of bray distances and 16S rRNA OTU relative abundance, respectively, at the phylum (a. & b.), family (c. & d.), and genus taxonomic ranks (e. & f.) of bacterial taxa, ≥ 1% relative abundance, associated with fish species.

3.2. Fish virome

Caudovirales phages, historically assigned based on virion morphology, show no clear delineation when using metagenomics and comparative sequence analysis. However, until an updated taxonomic structure is in place, we assessed the existing taxonomic family-level ranks of *Caudovirales* contig sequences detected in the intestinal contents of fishes as a method of measuring the diversity of phages detected. Similar levels of *Myoviruses*, *Podoviruses* and *Siphoviruses* were detected in the faeces of these marine fish (Figure 4.4a.; Kruskal-Wallis test, p = 0.406). With regards *Microviruses* (and *Circoviruses*) the relative abundances and observed frequencies should be interpreted cautiously, as 'random' amplification is known to preference single stranded DNA and small circular genomes (Kim and Bae 2011).

After trimming, there was an average of 2 481 766 (min. 971 545, max. 4 228 119) paired and 1 424 711 (min. 31 610, max. 4 082 498) unpaired high-quality reads per file. The number of reads aligning to the *de novo* detected phage contigs highlighted that each fish species examined had a unique phage composition to their viromes (Figure 4.4b.; PERMANOVA test, R^2 0.18, $p < 0.001$). The number of reads aligned to the RVDB database, which is predominantly composed of eukaryotic viruses, similarly resulted in a separation of viromes by fish species (Figure 4.4e.; PERMANOVA test, R^2 0.36, $p < 0.001$).

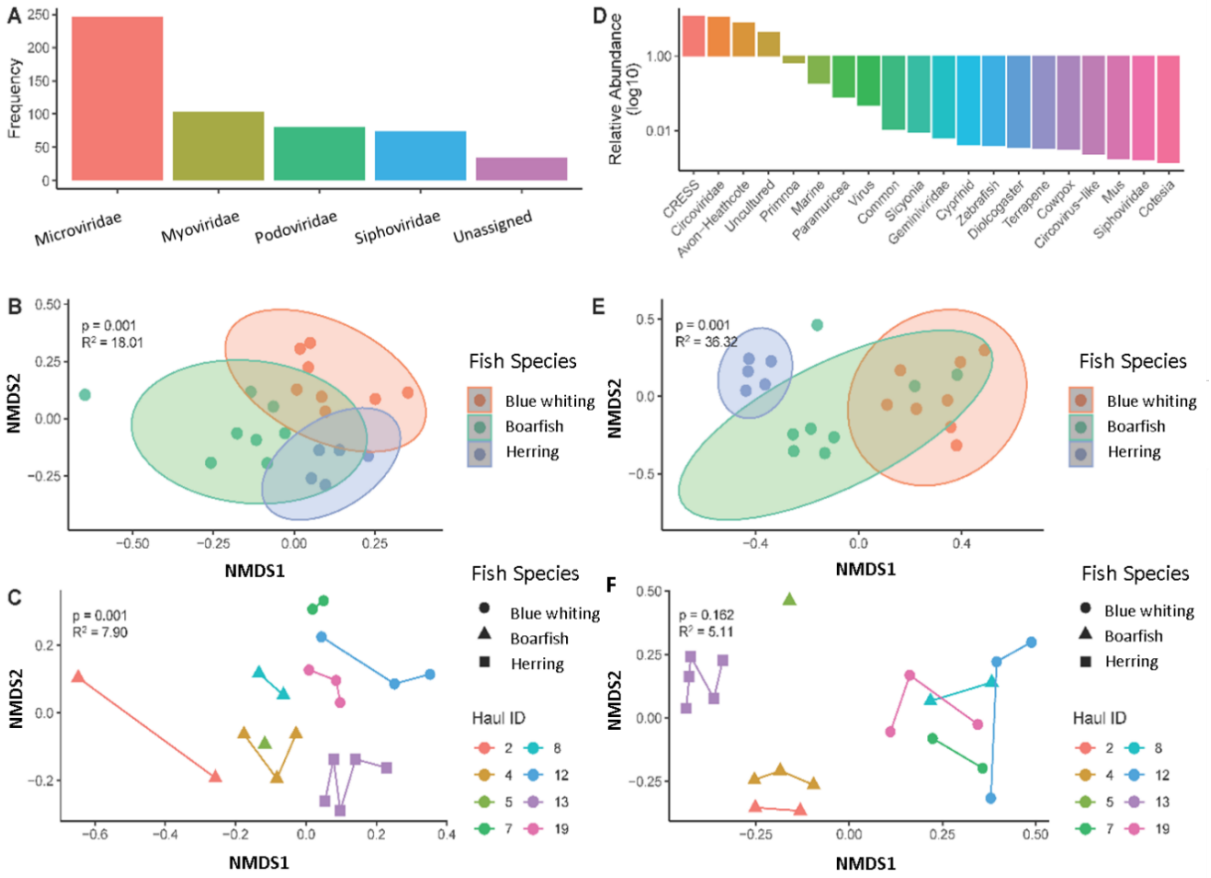

Figure 4.4. Prediction of phage and viral compositions within fish faeces. Abundance of *de novo* identified phage contigs assigned a putative family level taxonomic rank (a.). Ordination of the relative abundances of fish-associated phage compositions, grouped by fish species (b.) or connected by fishing haul site (c.). Relative abundance (log scaled) of reads mapped to the viral (eukaryotic and prokaryotic) RVDB database (d.). Ordination of the viral relative abundances detected against the RVDB database, grouped by fish species (e.) or connected by fishing haul site (f.).

3.2.1. Fish viral diversity

The average relative abundance of fish intestinal content phage contigs across different individual fish (mean = 0.016) highlights each fish has a unique phage community composition. This is also true within specific fish species, as boarfish, blue whiting and herring have mean relative abundances of *de novo* detected phage contigs of 0.011, 0.025, and 0.012, respectively. In the heatmap, produced from scaling relative abundance of assigned contig reads, high abundances form phylogenetically similar clusters in samples from the same species. It can be seen that the viruses found at high abundance in each fish species are usually completely absent in the other two fish species. This finding is especially evident in blue whiting samples (Figure 4.5.).

The diversity within the fish viromes was also assessed through phylogenetic analysis of abundant proteins. For *Caudovirales* phages, a comparison of the large terminase subunit was performed. As expected, there was no specific separation of terminase protein sequences by fish species (Figure 4.6a.). Interestingly, it is also clear that there is limited clustering of terminase sequences by family-level taxonomic prediction.

For *Microviridae* predicted phages, a comparison of capsid protein sequences was performed from fish only and with the addition of sequences from NCBI belonging to *Microviridae* characterised to the level of subfamily (Figure 4.6b. & 4.7.b., respectively). Few fish *Microviridae* clustered with *Alpavirinae* and *Bullavirinae* subfamily sequences, with most fish *Microviridae* clustering with *Gokushovirinae* and *Pichovirinae* as well as a new distinct branch outlined in the following section

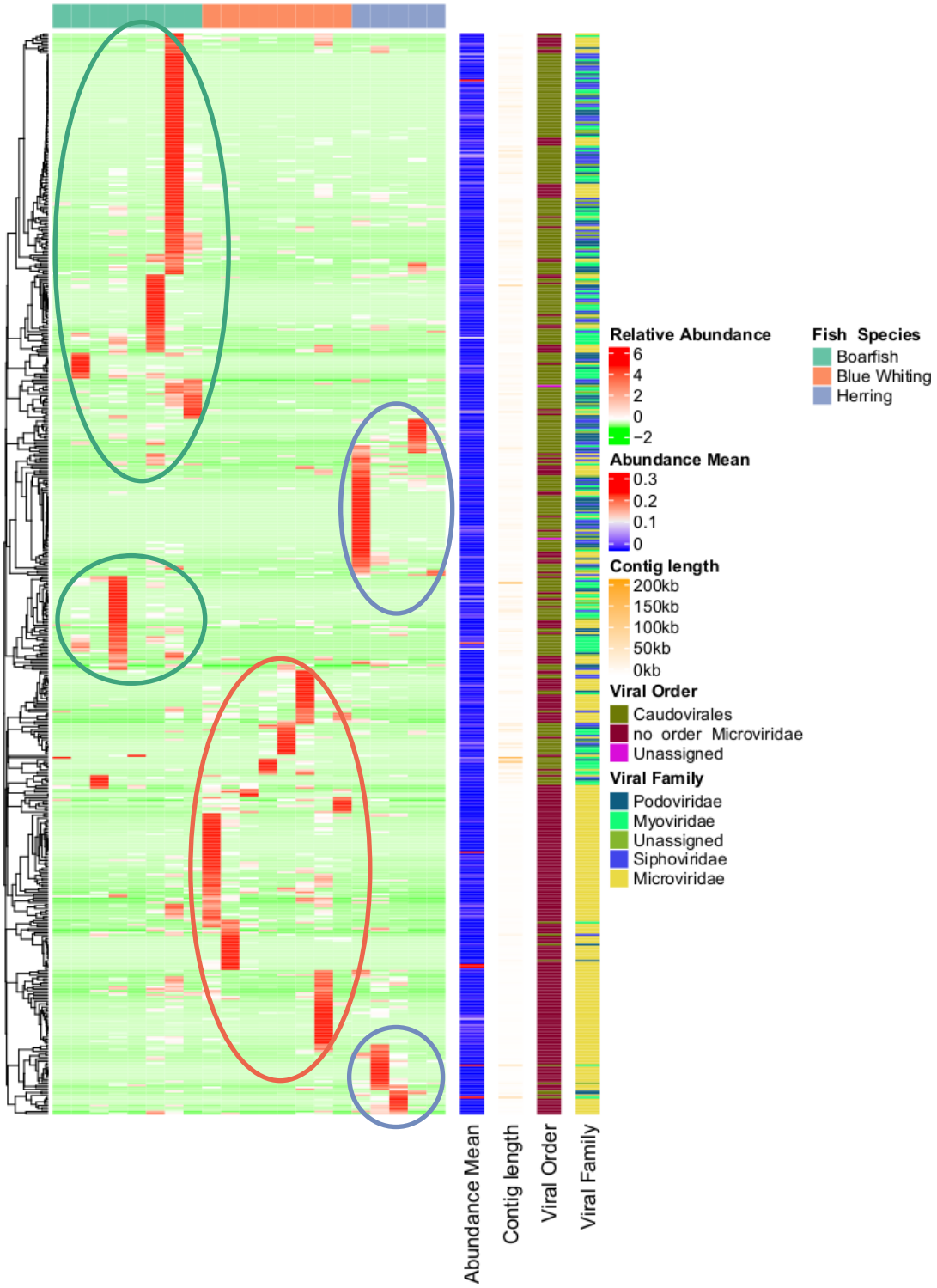

Figure 4.5. A heatmap displaying the relative abundance of *de novo* predicted phages associated with fish faecal viromes. Phylogenetic clustering is by rows. Phages at high abundances form phylogenetically similar clusters in samples from the same species (highlighted by circles). The uniqueness of each fish species virome is evident in this figure showing that phages found at high abundance in each fish species are usually completely absent in the other two fish species.

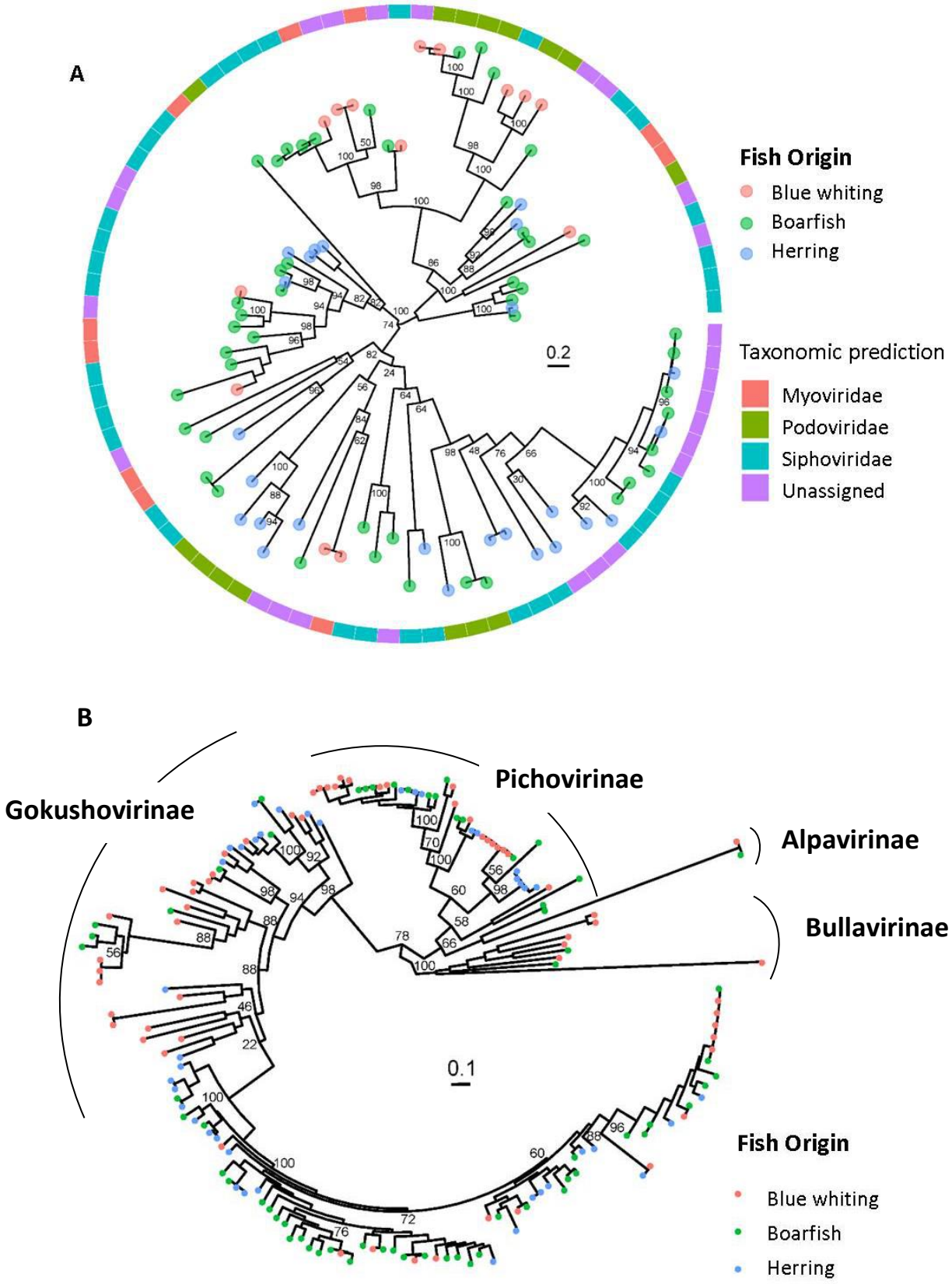

Figure 4.6. Phylogeny of abundant phage protein sequences within fish viromes. Comparison of *Caudovirales* large terminase subunit sequences (a.), *Microviridae* capsid sequences detected in fish viromes (b.) and *Circoviridae* replication protein sequences (c.).

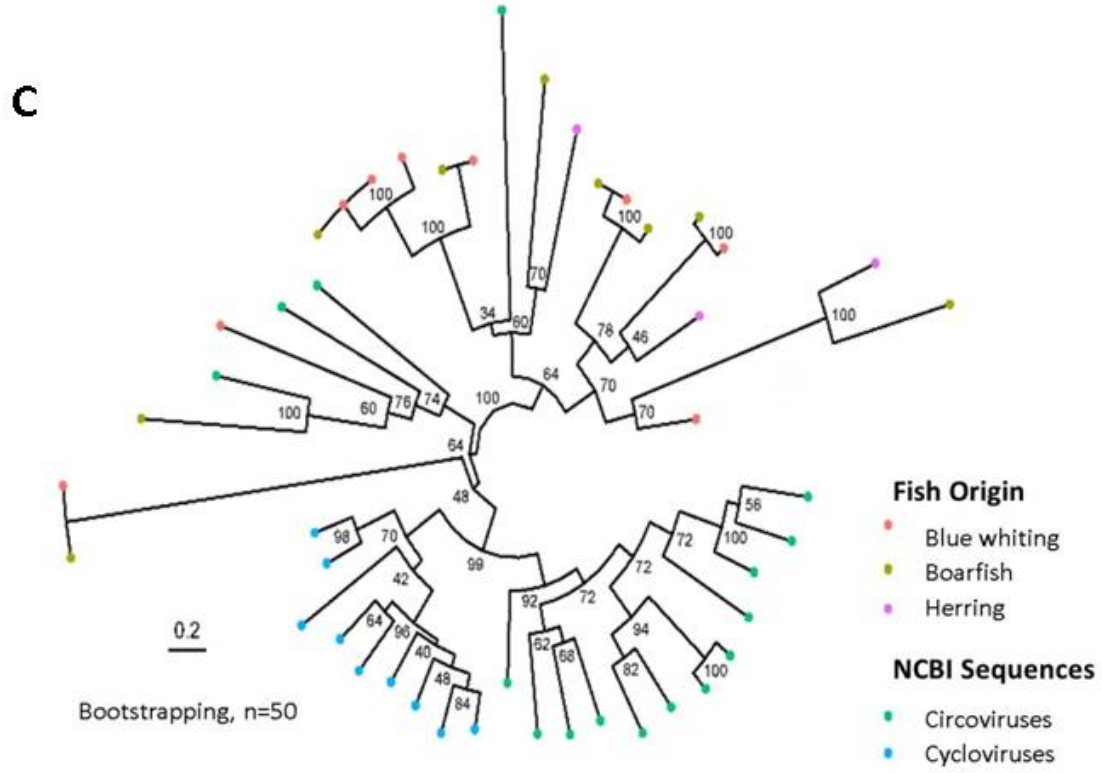

Figure 4.6. Phylogeny of abundant phage protein sequences within fish viromes. *Continued.*

3.2.2. Viral evolution

Numerous biogeographical studies have examined the phage composition of Earth's oceans, yet none (to our knowledge) have used metagenomics to examine the faecal viromes of marine fish. Therefore, to assess the similarity of fish faecal viromes with other environments, we compared the *Caudovirales* terminase sequences and *Microviridae* capsid sequences from fish faecal viromes with ocean studies and the human virome. Additionally, the replication protein sequence of *Circoviridae* from the three fish species was compared to those in the NCBI database, which originated from predominantly from mammals and bird species.

Caudovirales terminase sequences from the human gut virome formed two large clusters (highlighted with arrows), distinct from fish viromes and the ocean studies (Figure 4.7a.). Yet the relatedness of *Caudovirales* terminase sequences from fish viromes were intermingled with the Tara Ocean and North Sea study sequences, indicating the *Caudovirales* within this environment are more closely related.

Microviridae from fish faecal viromes were substantially different from those detected in the Tara Ocean virome study (Figure 4.7b.). While the *Gokushovirinae* subfamily had multiple *Microviridae* from both fish viromes and the Tara Ocean virome study, *Pichovirinae* was dominated by fish viral sequences only. Distinct clusters of fish associated *Microviridae* sequences are observed that contain neither subfamily characterised sequences nor sequences detected in other environmental or human studies.

The analysis of *Circoviridae* replication protein diversity, which includes NCBI sequences from two defined genera, resulted in a clear separation of NCBI characterised *Circoviridae* (predominantly from mammals and birds) from fish-associated sequences (Figure 4.7c.). In addition, *Circoviridae* replication protein sequences did not cluster by fish species, with high identity observed between *Circoviridae* sequences from differing fish viromes.

The evolutionary distance caused by single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) of a conserved *Microviridae* capsid sequence was also investigated (contig BF_45_21). In two instances, the dominant capsid allele associated with two different blue whiting, which differed by haul site, were found to be identical. When the dominant sequence allele of *Microviridae* capsid sequences detected per fish was investigated by fishing haul site, it was observed that in two samples there was clustering by sequence variants (Figure 4.8., shaded grey and gold). However, the *Microviridae* capsid

sequence of fish HR_1318 did not cluster together with other herring from haul 13 (shaded grey), while *Microviridae* capsid sequences of boarfish from haul 4 displayed high sequence divergence (connected by red dotted lines).

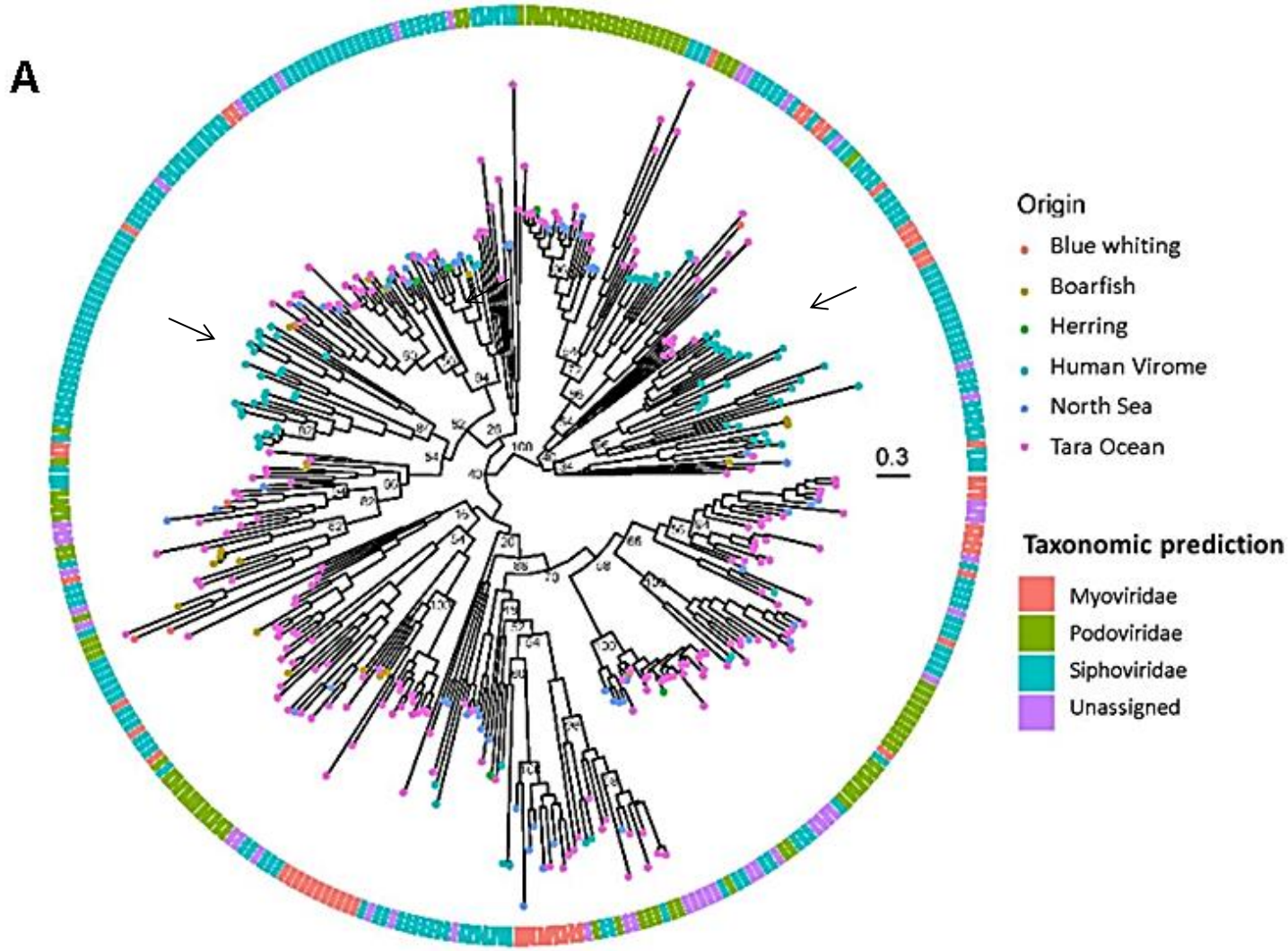

Figure 4.7. (a.) Phylogenetic comparison of abundant phage and viral protein sequences within fish viromes and the *Tara Ocean Virome*, the *North Sea goes viral* and the *Human Phageome* studies (highlighted with arrows). Comparison of *Caudovirales* large terminase subunit sequences. The annotation ring surrounding the terminase phylogenetic tree shows putative family-level taxonomic assignments.

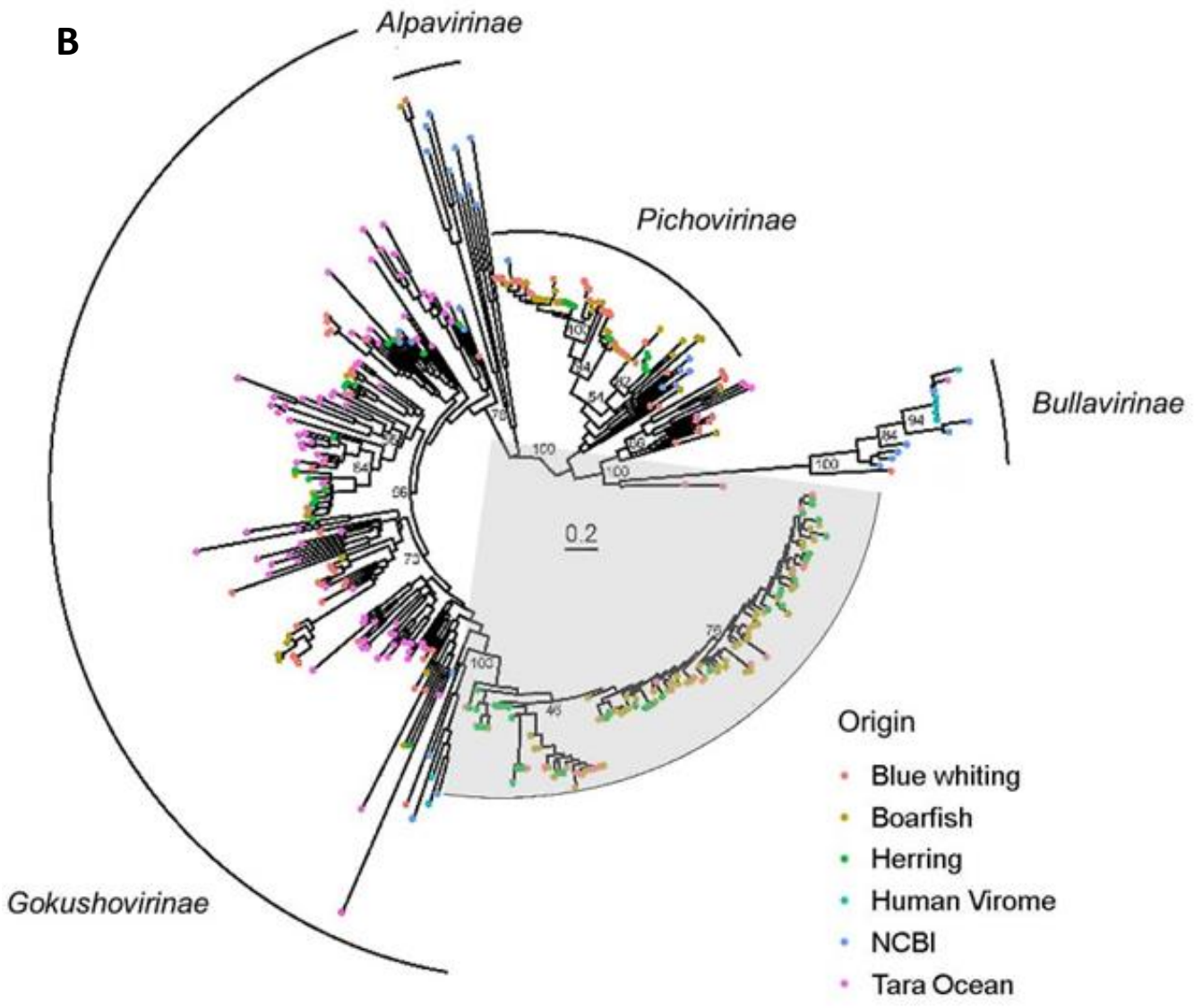

Figure 4.7. (b.) Phylogenetic comparison of abundant phage and viral protein sequences within fish viromes and the *Tara Ocean Virome*, the *North Sea goes viral* and *Human Phageome* studies (highlighted with arrows). Comparison of *Microviridae* capsid sequences. Subfamily taxonomic assignments to *Microviridae* were applied to all phylogram branches below the top node for which all NCBI sequences clustered under.

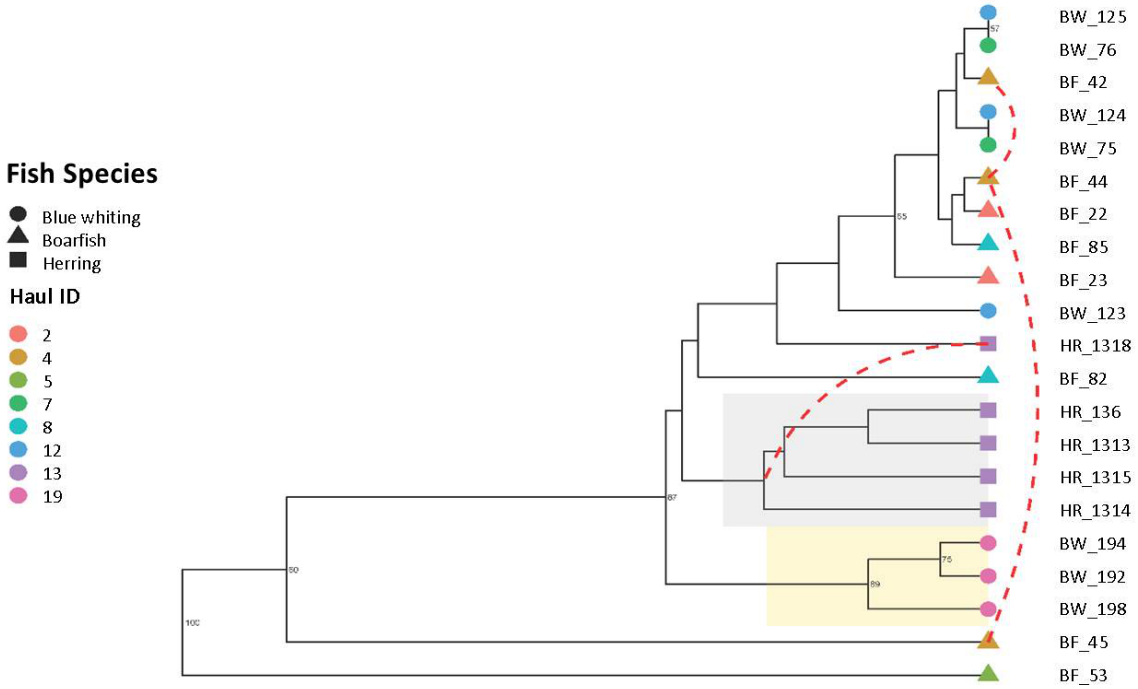

Figure 4.8. Cladogram visualisation of the phylogenetic distance caused by single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) of major capsid protein (MCP) encoding sequences of fish associated *Microviridae*. The evolution of *Microviridae* SNPs/indels, observed across MCP encoding sequences, demonstrate examples of homogeneity between fish caught at the same haul site (shaded grey and gold) and also heterogeneity between the same fish species caught at the same haul site (connected by red dotted lines).

4. DISCUSSION

The use of metagenomic shotgun sequencing to investigate the gut virome of three commercially important marine fish; blue whiting, boarfish and herring, produced large amounts of data and, although massive diversity existed, significant amounts of these sequences appeared to relate to novel viral elements. A lack of suitable phage reference genomes in public databases means that only a limited amount of phage data could be inferred from alternative studies. Indeed, Forde and Hill (2018) have suggested that even within the human phageome, on which the majority of studies in the past two decades have been published, more than 90% of phages are still unidentified.

From the data that could be assigned, it was found that *Microviridae* was the most abundant viral family. This was followed by the *Caudovirales* families, *Myoviridae*, *Podoviridae* and *Siphoviridae*, which were all found at similar abundance to each other. A recent study that used culture dependent enumeration techniques and transmission electron microscopy to quantify and classify viruses and prokaryotes within the gut contents of Tilapia (*Sarotherodon melanotheron*) also found relatively high numbers of *Caudovirales* and these were at significantly higher levels compared to in their surrounding aquatic environment (Bettarel *et al.* 2018). In a recent study of surface water phageomes in the North Sea, *Caudovirales* were by far the most dominant of the assigned groups, with *Myoviridae* as the most abundant family (Garin-Fernandez *et al.* 2018).

As discussed in the results, the relative high abundance of *Microviridae* detected should be viewed with caution. It has been well reported that the use of whole genomic amplification methods, such as multiple displacement amplification, prior to sequencing results in disproportionate amounts of short single-stranded circular genomic replication (Shkoporov *et al.* 2018). Within samples, viral DNA is in minute quantities in comparison to bacterial or other DNA. This is because viral genomes mostly range from about 0.02 to 0.06 Mb, compared to bacterial genomes which generally range between 1 – 13 Mb in size (Zhang and Gui 2018). As a result, contamination of phage fractions with bacterial DNA can pose difficulties during sequence analysis (Hayes *et al.* 2017). Whole genomic amplification prior to sequencing helps to reduce these difficulties.

Although phylogenetic assignment is limited for bacteriophage, comparisons of diversity and genetic composition between sample groups still provides informative data. In

this study, we have shown that the viromes of three species of marine fish caught in the same habitat are distinct and species specific. Principal coordinate analyses showed phage and virus compositions clearly separate by fish species and the average relative abundance of fish intestinal content phage contigs across different individual fish highlights how each fish has a unique phageome, a characteristic also found in human gut virome studies (Reyes *et al.* 2010; Shkoporov *et al.* 2019). Clustering of the relative abundances observed for the *de novo* detected phages, as seen in the heatmap in figure 4.5., further highlights species specificity of fish viromes. Phages found in high abundance in each fish are detected at low or absent levels in other fish and fish species.

Like the virome analysis, the bacteriome of these fish also clustered clearly by fish species at the phylum, family and genus levels. Phage and bacteria communities are inherently linked. The precise way in which this predator-prey dynamic exists is much postulated upon, with a number of models such as 'killing the winner', 'food-specialist', 'Red Queen dynamics', 'piggy-back the winner', 'host–virus arms race' and 'low trade-off between competition and defence' being proposed (Sandaa *et al.* 2009; Betts *et al.* 2014; Thingstad *et al.* 2014).

The bacteriomes of the three fish species varied significantly. Only two bacterial families (*Vibrionaceae* and *Lactobacillaceae*) were detected in the core microbiota of all three fish. The family *Vibrionaceae*, which is ubiquitous in aquatic environments, is composed of seven genera (*Vibrio, Photobacterium, Allomonas, Listonella, Enhydrobacter, Salinivibrio and Enterovibrio*; Nair *et al.* 2006). Of these there were only three (*Vibrio, Photobacterium* and *Enterovibrio*) that were found to exist at a relative abundance of ≥0.01% in blue whiting, boarfish and herring. *Vibrio* and *Photobacterium* are frequently cited as being the most dominant genera found in marine fish gut microbiota communities (Sullam *et al.* 2012; Egerton *et al.* 2018). Species within these genera are diverse in their host interactions. While many are pathogenic, others play important roles in digestion, working as symbionts producing digestive enzymes to aid in the breakdown of lipids, chitin and other dietary elements. Considering that the principle elements of the diet of all these fish are reported as chitinous-rich copepods (Cabral and Murta 2002; Langøy *et al.* 2012; Egerton *et al.* 2017), it is unsurprising that they all harbour these dominant, symbiotic, enzyme-producing bacteria. In line with this observation, many studies previously have found diet to be an important factor in influencing gut bacterial compositions of fish (Cordero *et al.* 2015; Schmidt *et al.* 2016; Zhou

et al. 2018). Host ecology is another prevailing factor of fish gut microbial composition (Wong and Rawls 2012). The spawning behaviours of these fish are a trait in which differences are seen. Considering the gut microbiota of fish develop from bacterial colonisation of eggs and the first feeding, it is reasonable to propose this as a likely influence of microbial divergence (Wang *et al.* 2018). With the ubiquitous distribution of phage in the marine environment, it is logical to propose that the initial virome is similarly determined.

Bacteriome and virome analyses were carried out on samples collected from different fish. Therefore, any comparisons made are speculative due to the multitude of confounding factors. However, it is interesting to note possible associations and relationships between the results that could guide future research in this area. The trends in 16S rRNA species richness (Chao1 α-diversity) seen in the three fish species is mirrored in the α-diversity of the phageomes. These results may suggest that bacterial species richness is correlated with the diversity of phage present. However, the understanding of actual infection networks and interactions is complex, and it is unlikely to simply be a one-to-one or modular network that exists. In fact, some recent peer-reviewed articles are suggesting a more intricate nested structure (Flores *et al.* 2011; Weitz *et al.* 2013; Koskella and Brockhurst 2014). Interestingly, the 16S rRNA Shannon index α-diversity did not correlate with the phageome diversity and these results actually align with the nested structure theory. For example, boarfish may harbour high levels of specialist phage that control the bacteriome to have many species at low levels (high species richness but lower diversity evenness) while blue whiting may harbour lower phage diversity but this may include greater numbers of generalists that prevent dominance and could increase 16S rRNA α- diversity. These theories would be interesting to explore in future work, comparing bacteriomes and viromes within the same host.

To delve deeper into the diversity and evolution within the fish viromes, phylogenetic analysis of abundant proteins was completed for a large terminase subunit encoded by many *Caudovirales* phages, capsid protein sequences in *Microviridae* and replication protein diversity in *Circoviridae*. Since the current taxonomy of *Caudovirales* phages are still based on virion morphology, sequences did not cluster into specific families. Also, specific separation of terminase protein sequences by fish species was not seen. However, it can be seen in the phylogenetic tree created from the large terminase subunit common in *Caudovirales* that boarfish contained a phylogenetically diverse virome spanning the entire tree while blue whiting and herring samples did not contain any sequences from entire branches.

A recent article discussing bacteriophage evolution presented evidence of two different evolutionary modes defined by the rate of horizontal gene transfer (Mavrich and Hatfull 2017). They reported that lytic phage gene content dissimilarity is roughly proportional to Mash (Ondov *et al.* 2016) distance (low gene content flux (LGCF) mode), and temperate phage can be split into two classes, one similar to lytic phage and a second in which gene content dissimilarity increases more quickly as Mash distance grows (high gene content flux (HGCF) mode). Therefore, viromes with high abundance of temperate phage will have increased genetic diversity. Furthermore, they highlighted that, contrarily from *Myoviridae* and *Siphoviridae*, all *Podoviridae* temperate phage were HGCF mode phage (Mavrich and Hatfull 2017). In the results from this study, *Podoviridae* are much more prevalent in the branches of the tree where the herring or blue whiting viromes are not represented. Moreover, almost double the numbers of *Podoviridae* are associated with the boarfish virome. Lastly, they revealed how phages infecting hosts within different phyla show different distributions among HGCF and LGCF modes. Phages of Bacteroidetes are almost exclusively HGCF mode, while those for Cyanobacteria are all LGCF mode. As for Proteobacteria, 50% of infecting temperate phages are HGCF mode, while this is true for over 80% of temperate phages infecting Firmicutes (Mavrich and Hatfull 2017). Considering this with the relative abundance bacterial composition of the three fish, it would be expected that boarfish (almost exclusively Proteobacteria) would have a high level of viral diversity, while herring (with a significant proportion of Cyanobacteria) may show lower genetic diversity in its virome. The phylogenetic diversity of *Caudovirales* phage in this study provides some support for such a trend. Aside from phage life mode, other factors such as microbial density, gene pool size, and environmental conditions will also influence genetic evolution (Letarov and Kulikov 2009; Norman *et al.* 2009; Knowles *et al.* 2016).

As well as diversity between the three different fish seen in the *Caudovirales* phages, comparison of the *Microviridae* and *Circoviridae* sequences found in the fish with those deposited on the NCBI database reveals the significant pool of novel genetic diversity existing in these viromes. The majority of the *Microviridae* detected in fish samples were novel and did not match with any sequences on the database. *Microviridae* is an important and highly diverse family of phage in which new entire subfamilies are still being discovered (Doore and Fane 2016; Creasy *et al.* 2018). *Circoviridae* are eukaryotic viruses that are known to cause fatal disease in birds and swine (Rosario *et al.* 2017). The first report of a circovirus in fish was

in 2011 (Lőrincz *et al.* 2011) and the ability of fish circoviruses to induce disease is not yet known (Tuboly 2016). The results from this study show a notable divergence of *Circoviridae* sequences of these marine fish viromes from the known cyclo- and circoviruses thus far, clearly depicting a gap in the knowledge of this potentially important pathogenic fish virus.

To further investigate the novelty of the fish virome, a comparison was made between the *Caudovirales* terminase sequences and *Microviridae* capsid sequences from fish faecal viromes with ocean studies and also the human virome. Unlike the human virome, which is mostly represented by a few clustering branches, *Caudovirales* from the fish virome are spread around the tree, dotted between sequences derived from the North Sea and Tara Ocean studies. This is also seen in the *Microviridae* analysis. Furthermore, the level of novel *Microviridae* sequences, unique to the fish virome, is remarkable. Significant fish linked *Microviridae* evolution appears to have taken place. The evolutionary distance caused by single nucleotide polymorphisms (SNPs) and insertion/deletions (indels) of a conserved *Microviridae* capsid sequence was also investigated. Sequence allele variants did not separate by fish species but did show some clustering in relation to sample haul site among blue whiting and herring samples, though not boarfish. This further highlights the diversity within the boarfish virome but also suggests some geographical effect. In numerous ocean virome studies geographic region or location has been found to be a significant driver in viral community structure (Flores *et al.* 2013; Hurwitz *et al.* 2014). However, the small sample sizes in this study make such interpretations speculative, and further investigation is warranted.

The gut microbiota composition of fishes are unique from their surrounding environment, shaped by host-specific selective pressures within the gut (Bevins and Salzman 2011). The results from this study highlight the enormous source of genetic diversity that exists within the virome of ocean-based fish species. These organisms are reservoirs of unique microbiomes. We show that both the fish bacterial microbiomes and viromes separate by species and suggest that this diversity may be shaped, at least in part, by the fish's diet and life history traits. While we recognise the limitations of this study in regards to the small sample sizes and the division of bacteriome and virome results between individuals, this study provides valuable initial data on the gut microbiome of these species and adds worthy results to an area of research that has received inadequate attention to date.

Acknowledgements

We would like to gratefully acknowledge the Teagasc Sequencing Facility, Dr Fiona Crispie, Dr Paul Cotter and Ms. Laura Finnegan for their technical assistance with the 16S rRNA MiSeq sequencing. Furthermore, we would like to acknowledge and thank Ms. Karen Daly and Dr Lorraine A. Draper for their guidance on virome extractions and sequencing and Mr. Rory Scarrott for producing the map of sample collection points in Figure 1. Finally, we would like to recognise the hard work of the scientists and crew on board the Celtic Explorer whose work permitted successful sample collection for this study. This work was supported by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. via the IRC Enterprise Partnership Scheme (EPSPG/2015/57 to S.E.), the Marine Institute and the Department of Agriculture, Food and the Marine (DAFM) in Ireland via the Sea Change Strategy, NutraMara programme (Grant-Aid Agreement No. MFFRI/07/01 to C.S.), and the SMARTFOOD project: 'Science Based 'Intelligent'/Functional and Medical Foods for Optimum Brain Health, Targeting Depression and Cognition' project (Ref No. 13/F/411 to C.S.) and Science Foundation Ireland in the form of a centre grant (APC Microbiome Ireland Grant No. SFI/12/RC/2273).

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SUPPLEMENTARY MATERIAL

Table S4.1. The core microbiota (only OTUs present in every sample at a relative abundance of ≥ 0.01% (Astudillo-García, Bell *et al.* 2017) of the intestinal contents of boarfish, blue whiting and herring, at the Family taxonomic level, derived from 16S rRNA compositional data. Mean ± SD.

Phylum	Class	Order	Family	Boarfish (%)	Blue whiting (%)	Herring (%)
Actinobacteria	Actinobacteria	Actinomycetales	*Propionibacteriaceae*			0.05 ± 0.04
Bacteroidetes	Bacteroidia	Bacteroidales	*Rikenellaceae*		14.73 ± 7.01	0.76 ± 0.68
Cyanobacteria	Cyanophyceae	Synechococcales	*Synechococcaceae*			8.00 ± 6.08
Deferribacteres	Deferribacteres	Deferribacterales	*Deferribacteraceae*		5.41 ± 3.55	
Euglenozoa	Euglenoidea	Euglenales	*Euglenaceae*			6.44 ± 8.13
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	2.77 ± 2.07	0.38 ± 0.78	7.83 ± 5.93
Firmicutes	Clostridia	Clostridiales	*Ruminococcaceae*		1.90 ± 1.10	
Foraminifera	Globothalamea	Rotaliida	*Virgulinellidae*			1.29 ± 1.31
Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	0.31 ± 0.24		2.53 ± 2.54
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*			0.07 ± 0.08
Proteobacteria	Alphaproteobacteria	Rhodobacterales	*Rhodobacteraceae*			0.08 ± 0.10
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	*Desulfovibrionaceae*		4.81 ± 3.98	0.17 ± 0.18
Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Hahellaceae*	9.34 ± 11.65		0.26 ± 0.29
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*			0.21 ± 0.21
Proteobacteria	Gammaproteobacteria	Vibrionales	*Vibrionaceae*	76.22 ± 15.60	29.68 ± 23.51	28.15 ± 23.20
Spirochaetes	Spirochaetia	Brachyspirales	*Brachyspiraceae*		12.34 ± 5.68	0.77 ± 0.89
Spirochaetes	Spirochaetia	Brevinematales	*Brevinemataceae*		16.40 ± 8.00	1.36 ± 1.56
Spirochaetes	Spirochaetia	Spirochaetales	*Spirochaetaceae*			1.50 ± 1.45
Tenericutes	Mollicutes	Entomoplasmatales	*Mycoplasmataceae*		8.54 ± 7.12	24.57 ± 30.75

Table S4.2. The core microbiota (only OTUs present in every sample at a relative abundance of ≥ 0.01% (Astudillo-García, Bell *et al.* 2017) of the intestinal contents of boarfish, blue whiting and herring, at the Genus taxonomic level, derived from 16S rRNA compositional data. Mean ± SD.

Phylum	Class	Order	Family	Genus	Species	Boarfish (%)	Blue whiting (%)	Herring (%)
Bacteroidetes	Bacteroidia	Bacteroidales	*Rikenellaceae*	*Alistipes*				0.05 ± 0.04
Bacteroidetes	Bacteroidia	Bacteroidales	*Rikenellaceae*	*Rikenella*			14.05 ± 6.76	0.70 ± 0.65
Cyanobacteria	Cyanophyceae	Synechococcales	*Synechococcaceae*	*Synechococcus*				8.00 ± 0.06
Deferribacteres	Deferribacteres	Deferribacterales	*Deferribacteraceae*	*Mucispirillum*			5.41 ± 3.55	
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*		2. 77 ± 2.07		7.83 ± 5.93
Firmicutes	Clostridia	Clostridiales	*Ruminococcaceae*	*Anaerotruncus*			1.16 ± 0.78	
Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	*Blastopirellula*		0.08 ± 0.06		0.54 ± 0.50
Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	*Rhodopirellula*				0.74 ± 1.01
Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	*Rubripirellula*		0.10 ± 0.07		0.77 ± 0.71
Planctomycetes	Planctomycetia	Planctomycetales	*Planctomycetaceae*	*uncultured*				0.33 ± 0.24
Proteobacteria	Alphaproteobacteria	Rhizobiales	*Rhizobiaceae*	*Rhizobium*				0.07 ± 0.08
Proteobacteria	Deltaproteobacteria	Desulfovibrionales	*Desulfovibrionaceae*	*Desulfovibrio*			4.74 ± 4.05	0.17 ± 0.18
Proteobacteria	Gammaproteobacteria	Oceanospirillales	*Hahellaceae*	*Endozoicomonas*		9.34 ± 11.65		0.26 ± 0.29
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*	*Acinetobacter*				0.08 ± 0.06
Proteobacteria	Gammaproteobacteria	Pseudomonadales	*Moraxellaceae*	*Psychrobacter*				0.08 ± 0.06
Proteobacteria	Gammaproteobacteria	Vibrionales	*Vibrionaceae*	*Enterovibrio*		27.91 ± 24.22	9. 16 ± 21.95	0.26 ± 0.38
Proteobacteria	Gammaproteobacteria	Vibrionales	*Vibrionaceae*	*Photobacterium*		42.49 ± 28.64	17.65 ± 9.85	20.43 ± 23.09
Proteobacteria	Gammaproteobacteria	Vibrionales	*Vibrionaceae*	*Vibrio*		5.75 ± 5.75	2.51 ± 3.02	2.56 ± 2.56
Spirochaetes	Spirochaetia	Brachyspirales	*Brachyspiraceae*	*Brachyspira*			12.34 ± 5.68	0.77 ± 0.89
Spirochaetes	Spirochaetia	Brevinematales	*Brevinemataceae*	*Brevinema*			16.40 ± 8.00	1.36 ± 1.56
Spirochaetes	Spirochaetia	Spirochaetales	*Spirochaetaceae*	unclassified				1.46 ± 1.38
Tenericutes	Mollicutes	Entomoplasmatales	*Mycoplasmataceae*	unclassified				23.31 ± 31.64
Euglenozoa	Euglenoidea	Euglenales	*Euglenaceae*	*Euglena*	*mutabilis*			6.44 ± 8.13
Foraminifera	Globothalamea	Rotaliida	*Virgulinellidae*	*Virgulinella*	*fragilis*			1.29 ± 1.31

Chapter 5

Characterization of protein hydrolysates from blue whiting (*Micromesistius poutassou*) and their application in beverage fortification

Published: *Food Chemistry* (2017), 245: 698-706.
doi: 10.1016/j.foodchem.2017.10.107

ABSTRACT

Enzymatic hydrolysis of fish proteins has been employed as a principle method for converting under-utilised fish into valuable products for the pharmaceutical and health food industries. In this study, six commercial enzymes were tested for their ability to make fish protein hydrolysate powders from whole blue whiting. The chemical and functional properties of these powders were compared. The powders all had high solubility (> 80%) across a wide pH range in water and their solubility improved further within a vitamin-tea beverage matrix (> 85%). Varying degrees of antioxidant activities were recorded for the powders using three model systems (DPPH, ferrous chelating and reducing power). This study demonstrates that commercial enzymes are useful for the extraction and alteration of fish protein from a low value source to produce highly digestible, low molecular weight peptide powders that could be used as a fortifying health ingredient, especially in beverages.

1. INTRODUCTION

The demand for protein is growing; driven by a rising population, changing food preferences and a growing recognition of the importance of protein as a key ingredient for health and nutrition. Dietary protein supplementation is becoming popular, especially for people on restrictive diets, athletes and the elderly. Dairy and soy are the main sources of protein used in nutritional beverage products thus far, with whey protein concentrate at 80% protein being the most widely used (Galaz 2014). However, fish is an excellent source of protein. It has higher protein content than most terrestrial animals and proven satiating effects (Egerton *et al.* 2017). Furthermore, fish proteins are highly digestible and have excellent essential amino acid (EAA) profiles that closely approximate human dietary requirements as set by the World Health Organisation (WHO). Many smaller fish species, e.g. blue whiting (*Micromesistius poutassou*), boarfish (*Capros aper*), and sprat (*Sprattus sprattus*), are currently considered undesirable for direct human consumption. These fish, often caught in vast numbers, are most commonly used in animal feed and fertilisers. However, they represent an untapped resource of functional and health promoting ingredients for human nutrition (Thorkelsson and Kristinsson 2009).

Enzymatic hydrolysis has been employed as a principle method for converting under-utilised fish into valuable products for the pharmaceutical and health food industries (Chalamaiah *et al.* 2010). This process can create products that provide enhanced bioactivity, nutritional and physicochemical properties beyond that of the parent protein (Ryan *et al.* 2011). The production of fish protein hydrolysates (FPH) has been widely studied and research shows that the properties of the hydrolysates are highly dependent on the hydrolytic process and reaction conditions, as well as the specific substrates and enzymes used (Kristinsson and Rasco 2000b; Chalamaiah *et al.* 2012). Previous studies of FPH have shown that, when added to food, they can contribute to water holding, emulsification and texture properties (Halim *et al.* 2016). Enhanced solubility is a frequently reported and valued property of FPH (Benjakul *et al.* 2014). Bioactive properties such as anti-oxidation, anti-bacterial, anti-hypertension and anti-proliferation are also frequently reported (Picot *et al.* 2006; Thiansilakul *et al.* 2007; Lee *et al.* 2010; Song *et al.* 2011) Antioxidant properties can be an important feature for food preservation, as well as providing potential health benefits (Mendis *et al.* 2005; Phanturat *et al.* 2010).

Recently, some studies have gone beyond producing and characterizing FPH, and have tested their applicability to fortify foods. Mohamed *et al.* (2014) used carp and shark fish protein concentrate to increase protein content of biscuits without negatively affecting acceptability in sensory analyses. Similarly, positive results were provided by a sensory panel tasting cheese sticks and biscuits fortified with tilapia FPH up to 15% (w/w) (Ariyani and Widyasari 2000). Fish and roe PH have been successfully used to stabilize emulsions and protect against oxidation, especially with fish oil-water emulsions (Jónsdóttir *et al.* 2016; Ghelichi *et al.* 2017). These properties have been harnessed in the production of catfish sausages which resulted in finer fat globules and retardation of lipid oxidation during 12 days of storage (Intarasirisawat *et al.* 2014). The possibility of fortifying beverages with FPH is also beginning to be investigated. Galactose-gelatin hydrolysate from unicorn leatherjacket skin was used to fortify instant coffee brew, creating a source of antioxidant without negatively affecting sensory properties (Karnjanapratum and Benjakul 2017), while Chuaychan *et al.* (2017) have analysed the effects of different spray drying temperatures on the properties of gelatin hydrolysate from spotted golden goatfish scales with the aim of fortifying apple juice.

Blue whiting are fish caught in extensive shoals around the world. However, these fish are used primarily for fishmeal and are not considered palatable for direct human consumption. There have been few studies on producing hydrolysates from blue whiting, but they have presented results that show excellent potential for adding value to this resource. Hydrolysates made from isolated proteins from headed and gutted blue whiting with Alcalase 2.4 L were bioactive with up to 75% angiotensin I-converting enzyme (ACE) inhibitory activity (Geirsdottir *et al.* 2011). In another study, it has been shown *in vitro* and subsequently *in vivo*, that blue whiting protein hydrolysates, produced using a mix of commercial enzymes, have satiating effects (Cudennec *et al.* 2012). Powders with properties such as these would be considered desirable as functional ingredients in food products.

This study adds to the research on blue whiting protein hydrolysates (BWPH). It aims to compare the applicability of six commercial enzymes for the production of BWPH. The objective is to characterize the peptide powders, comparing their chemical, functional and sensory properties and to assess their potential use in fortifying a vitamin tea beverage.

2. MATERIALS AND METHODS

2.1. Materials

Blue whiting was kindly donated by Biomarine Ingredients Ireland Ltd. The fish were caught in spring 2016 off the north-west coast of Ireland. They were transported frozen, on ice to the laboratory in March 2016 and were stored at −20 °C until required. Whole fish were homogenized (Robot Coupe blixer 2 commercial food processor, Stephens Catering Equipment Co. Ltd., Ireland) to create a uniform mince. The mince was stored in aliquots at -20 °C and defrosted as required prior to hydrolysis.

2.2. Chemicals and reagents

The enzymes Alcalase® 2.4 L (endoproteinase from *Bacillus licheniformis*), Protamex® (endoproteinase from *Bacillus* sp.), Savinase® 16 L (endoproteinase from *Bacillus* sp.), Neutrase® 0.8 L (endoproteinase from *Bacillus amyloliquefaciens*), Flavourzyme® 500 L (endoproteinase and exopeptidase from *Aspergillus oryzae*) and Papain (endoproteinase extracted from latex of the plant *Carica papaya*) were purchased from Sigma-Aldrich Ireland Ltd. and were food grade. All the chemicals used in different analyses were at least of analytical grade.

2.3. Enzyme activity assay

To evaluate and standardize the general proteolytic activity of the six enzymes, a synthetic substrate, Azocoll™ (Calbiochem-Novabiochem, La Jolla, CA) was used as described previously, with some modifications (Chavira *et al.* 1984; Kristinsson and Rasco 2000c). The assay was performed for the six enzymes at their optimum pH and temperature as outlined in the literature (Alcalase® 2.4 L: 50 °C, pH 8; Protamex®: 55 °C, pH 7.5; Savinase® 16 L: 50 °C, pH 9; Neutrase® 0.8 L: 50 °C, pH 6.5; Flavourzyme® 500 L: 50 °C, pH 7; Papain: 65 °C, pH 6.5). To begin, Azocoll (75 mg) was mixed with 0.1 M sodium phosphate buffer (pH 7.5) and left to stand for one hour before centrifuging (27670 *g*, 40 °C, 2 min) and removing supernatant. The step was repeated once more. This removed excess small azo-dye labelled collagen peptides that can interfere with the assay. Sodium phosphate buffer (1.5 mL), adjusted to optimum pH for each enzyme, was added to the Azocoll™ and heated for five min. Enzyme (10 µL) was added to the assay tubes at five levels of increasing concentration. After mixing, the tubes

were incubated at optimum temperature for 15 min. The reaction was terminated by putting the tubes on ice for five min. The suspension was then filtered (Sarstedt Filtropur S 0.45) and the filtrate absorbance measured at 520 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). The absorbance was plotted against enzyme quantity used for each assay. The results were expressed as Azocoll units (AzU) per gram (g) of enzyme preparation, where one AzU is defined as the amount of enzyme producing absorption of 0.1 at 520 nm under the conditions described above.

2.4. Enzymatic hydrolysis

The hydrolysis reaction was performed using pH-stat apparatus (Titrando 842, Metrohm) equipped with Tiamo 1.1 software. Mince (100 g) was mixed with distilled water (1:2 w/v). The reaction pH and temperature were set at optimum for each enzyme (outlined in Section 2.3.) and the pH was kept constant using 1 N NaOH. Enzyme quantities equating to 39054 AzU, which corresponded to 0.1 mL of Alcalase, were used in the hydrolysis and the solution was stirred at 300 rpm throughout the reaction. Aliquots (3 mL) of the reaction mixture were removed at 10 intermittent time points (see Figure 5.1.). Following enzyme denaturation (20 min at 90 °C), 50 µL of the aliquots were added to 950 µL of 1% (w/v) sodium dodecyl sulfate (SDS) solution and stored at −20 °C until required for analysis. The soluble protein fractions of the main reaction products were isolated by centrifugation (4566 *g*, 0 °C, 20 min) and filtration (Whatman paper No. 2) and stored at −20 °C until required for freeze drying. The five resulting blue whiting soluble protein hydrolysate (BWSPH) powders are hence forth referred to by the name of their producing enzyme, e.g. powder of Alcalase 2.4 L is referred to as Alcalase hydrolysate.

2.5. Degree of hydrolysis

The trinitrobenzenesulfonic acid (TNBS) method was used with some modifications to calculate the degree of hydrolysis (DH). BWSPH time point samples, diluted in 1% SDS solution (0.15 mL) were added to tubes containing sodium phosphate buffer (1.0 mL, pH 8.2). A 5% TNBS solution (1.0 mL) was added; each tube was capped, mixed and incubated (60 min at 50 °C). The reaction was terminated by the addition of 0.1 N HCl (2 mL). Absorbance values were measured at 340 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). Results were

calculated using a standard curve prepared with L-leucine using the same methods. DH (%) was calculated using the following equation:

$$DH\% = \frac{AN_2 - AN_1}{Npb} \times 100$$

where AN_1 and AN_2 are the amino nitrogen content of the protein substrate before and after hydrolysis, respectively, and *Npb* is the nitrogen content of the peptide bonds in the protein substrate; taken as 123.3.

2.6. Proximate composition

Proximate composition was determined for whole blue whiting and the BWSPH. Moisture content was determined gravimetrically after drying at 105 °C for 24 h. Ash content was measured by incinerating the samples overnight in a furnace at 550 °C. Total nitrogen content was determined by the macro-Kjeldahl method and crude protein content was estimated by multiplying total nitrogen content by 6.25. Lipid content was determined gravimetrically after extraction following a modified Bligh and Dyer method (Smedes 1999). Protein and fat analyses were performed in duplicate.

2.7. Total and free amino acids

Free amino acid content was determined for the BWSPH according to McDermott *et al.* (2016). Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column. For total amino acid content of BWSPH, proteins were hydrolysed in 6 N HCl at 110 °C for 23 h and the resulting hydrolysates analysed as per free amino acid method.

2.8. Molecular weight distribution profile

Liquid BWSPH time point samples were diluted in distilled water (1:10 v/v) and filtered (Sarstedt Filtropur S 0.2). Molecular weight distributions were determined by size exclusion gel permeation chromatography using a high-performance liquid chromatography system (Waters Corporation, Milford, Massachusetts, USA) and a UV detector. Samples were injected (20 µL) on a TSK2000 SW (300 × 7.5 mm) and a TSK G2000swxl column in series (300 × 7.8 mm, Tosu Hass, Japan) using 30% (v/v) acetonitrile with 0.1% (v/v) trifluoroacetic acid buffer,

at a flow rate of 1 mL min^{-1}, as the eluent. Data were collected and analysed using a Waters Empower 3 software package. The column was calibrated with standard proteins dissolved at 1 mg mL^{-1} in distilled water and elutes were monitored at 214 nm. The percentage of individual protein fractions was calculated using the area under the peak of each component divided by the total area.

2.9. Functional properties

2.9.1. Solubility

Nitrogen solubility was initially determined in distilled water over a range of pH values (2, 4, 6, 8, 10) as described elsewhere (Sila *et al.* 2014). BWSPH (200 mg) were suspended in distilled water (30 mL) and mixed for 30 min at room temperature prior to centrifugation (4566 *g*, 20 °C, 20 min) and filtered (No. 2 Whatman filter paper). The nitrogen content of the resulting supernatant was determined using the Kjeldahl method and nitrogen solubility was calculated as follows:

$$Nitrogen\ solubility\ (\%) = \frac{N_1}{N_0} \times 100$$

where N_1 = supernatant nitrogen concentration and N_0 = sample nitrogen concentration.

Subsequently, nitrogen solubility of the BWSPH was determined in a vitamin-tea beverage following the method above. The pH of the beverage was measured. All analyses were performed in duplicate.

2.9.2. Emulsifying properties

The emulsion activity index (EAI) and emulsion stability index (ESI) of BWSPH were determined with some modifications (Sila *et al.* 2014). BWSPH were reconstituted in distilled water (15 mL) at 0.5%, 1% and 2% (w/v). Olive oil (5 mL) was homogenized with the BWSPH solution with a Tissue Tearor (Biospec Products, Model 985370) for one min at room temperature. Aliquots (50 µL) of the emulsion were taken from the bottom of the conical flask directly after the homogenization and again 10 min later and diluted 100-fold in 0.1% (w/v)

SDS solution. The new mixture was mixed for 10 s and the absorbance was measured at 500 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). The EAI and ESI were calculated as follows:

$$EAI\ (m^2 g^{-1}) = \frac{2\ \times 2.303\ \times\ A_0}{0.25\ \times protein\ weight\ (g)}$$

$$ESI\ (min) = \frac{\Delta A}{A_0} \times t$$

where A = absorbance, $\Delta A = (A_0 - A_{10})$ and t = 10 min.

2.9.3. Oil binding capacity

To analyse the oil binding capacity (OBC) of BWSPH, an aliquot (0.5 g) was weighed in a centrifuge tube and the combined weight (sample + tube) noted. Olive oil (10 mL) was added to the tube and kept at room temperature for one hour, mixing every 15 min for 5 s. The mixture was then centrifuged (2,000 *g*, 20 °C, 25 min), the supernatant decanted and the tube and sample re-weighed. OBC was calculated as the weight of the contents of the tube after draining, divided by the weight of the BWSPH sample and expressed as the% weight of dried BWSPH.

2.10. Antioxidant properties

2.10.1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity assay

The radical-scavenging activity of BWSPH was determined using DPPH based on previous methods (Wu *et al.* 2003). Briefly, 2.0 mL of sample (0.5, 1.5, 3, 6, 12, 18 mg mL^{-1}) was mixed with 2.0 mL of 0.15% (w/v) DPPH dissolved in 95% ethanol. The mixture was then kept at room temperature in the dark (30 min) before the reduction of DPPH radical was measured at 517 nm using a CARY 1 UV–vis spectrophotometer (Alto, CA). Distilled water was used as the negative control and butylated hydroxytoluene (BHT) (10 mM) was used as a positive control. The DPPH radical-scavenging activity was calculated as follows:

$$Scavenging\ ability\ (\%) = \left(A_c - \frac{A_1 - A_0}{A_c}\right)$$

where A_c is the absorbance of the negative control, A_1 is the absorbance of the sample mixed with the reagents and A_0 is the absorbance of the diluted samples without reagents.

2.10.2. Ferrous chelating assay

The Fe^{2+} chelating activity of BWSPH was determined according to Wang, Jónsdóttir, and Ólafsdóttir (2009). An aliquot (500 µL) of sample (0.5, 1.5, 3, 6, 12, 18 mg mL^{-1}) was mixed with 50 µL of 2 mM Iron (II) chloride and 1.6 mL of distilled water. After incubating at room temperature for 15 min, 100 µL of 5mM ferrozine was added and the mixture was left to stand for a further 10 min, after-which the absorbance was measured at 562 nm (CARY 1 UV–vis spectrophotometer; Alto, CA). Distilled water was used as the negative control and EDTA (100 µM) was used as a positive control.

$$Chelating\ activity\ (\%) = \left(A_c - \frac{A_1 - A_0}{A_c}\right)$$

where A_c is the absorbance of the negative control, A_1 is the absorbance of the sample mixed with the reagents and A_0 is the absorbance of the diluted samples without reagents.

2.10.3. Reducing power assay

Reducing power was measured according to the method of Gringer, Osman, Nielsen, Undeland, and Baron (2014). Diluted samples (0.5, 1.5, 3, 6, 9, 12 mg mL^{-1}) were centrifuged (14,000 *g*, 3 min, 20 °C). The supernatant (200 µL) was then mixed with 200 µL of 0.2 M phosphate buffer (pH 6.6) and 200 µL of 1% (w/v) potassium ferricyanide and incubated for 20 min at 50 °C. Subsequently, 200 µL of 10% (w/v) trichloroacetic acid was added, mixed and centrifuged (10,000 *g*, 10 min). In a microplate, 100 µL of the upper supernatant was mixed with 100 µL of distilled water and 20 µL of 0.1% (w/v) ferric chloride. The microplate was incubated for 10 min at room temperature before the absorbance was measured at 700 nm using Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT). Distilled water was used as the negative control and ascorbic acid (500 µM) was used as a positive control. The absorbance of the diluted samples, without reagents was also measured and results were

taken as the difference between the absorbance of the sample mixed with the reagents and the absorbance of the diluted samples without reagents. Results were expressed as OD_{700}.

2.11. Colour

Aliquots of BWSPH were diluted (200 mg mL^{-1} and 1000 mg mL^{-1}) and colour measurements were taken from the solutions in sealed glass bottom petri dishes (µ-Dish 35 mm; ibidi GmbH). Three repeated measures of L∗ (lightness), a∗ (red-green colour), and b∗ (yellow-blue colour) values were taken at random locations across the lid of the petri dishes using a Minolta Chroma-Meter CR-400 (Mason Technology Ltd., Dublin, Ireland). The mean of three repeated measures was calculated and used as a unit for one replicate.

2.12. Statistical analysis

All analyses were performed in triplicate unless otherwise stated. Statistical tests were performed using Microsoft Excel® and the SPSS® computer programs (SPSS Statistical Software, Inc., Chicago, IL.). All data were submitted to Analysis of Variance (ANOVA) and pairwise comparisons were conducted by Tukey's test. Significance level was determined at the 95% probability level.

3. RESULTS AND DISCUSSION

3.1. Enzyme activity assay

An activity assay was performed before enzymatic hydrolysis, as a method of estimating the quantity of each enzyme needed to obtain a uniform level of proteolytic activity. The results showed that Alcalase 2.4L (390541.06 AzU mL^{-1}), Protamex (250110.80 AzU mL^{-1}) and Savinase 16 L (177813.67 AzU mL^{-1}) had over 100-times greater activity than Neutrase 0.8 L (1394.74 AzU mL^{-1}), Papain (1225.08 AzU mL^{-1}) and Flavourzyme 500 L (6176.00 AzU mL^{-1}). The activity level recorded for Alcalase 2.4 L is within the same magnitude as reported elsewhere (Kristinsson and Rasco 2000c). Flavourzyme was also analysed by Kristinsson and Rasco (2000c) who reported an activity 10 times greater than what was recorded here. Although this difference is noteworthy, they worked with Flavourzyme 1000 L rather than Flavourzyme 500 L and the assays were performed under different pH and temperature conditions. Papain had the lowest activity level and a considerably higher cost (approximately 30 times more) compared with the microbial enzyme products. This made it unsuitable for commercial hydrolysate production and consequentially was not used in further experiments for this study. All other enzymes analysed were of fungal or microbial origin. Microbial derived enzymes have become prolific and now account for approximately 40% of the total worldwide enzyme sales (Rao *et al.* 1998). This is because microbes can be cultivated rapidly, in limited space and are easily manipulated to produce large quantities of desirable enzymes.

3.2. Degree of hydrolysis

DH is defined as the percentage of peptide bonds cleaved and it is the standard parameter commonly used to monitor and compare the level of proteolysis. The TNBS method was favoured over the other two common methods, the pH-stat and OPA methods, to calculate DH because previous studies have shown that it provides the most accurate results for a wide range of conditions (Spellman *et al.* 2003).

Based on the TNBS method, the hydrolysis curves of whole blue whiting after 3 h of reaction time with the five enzymes (at volumes dictated by the precluding activity assay) are shown in Figure 5.1. Statistically significant differences in DH were found between the enzymes ($F(4, 10) = 92.88$, $p < 0.001$). Flavourzyme produced a significantly higher DH compared to the other four enzymes (41.47 ± 0.76%; $p < 0.001$). Neutrase (22.78 ± 2.62%)

produced the second highest DH but was only considered significantly higher than that of Protamex which had the lowest final DH (16.43 ± 1.61%; $p < 0.05$). Comparing the DH across the three hours, an initial rapid rate of hydrolysis was observed in the first 20 min followed by a decreasing rate of hydrolysis. This is an archetypical reaction commonly observed (Kristinsson and Rasco 2000c; Gbogouri *et al.* 2004). Flavourzyme, however, continued to have greater increases in DH until 90 min of hydrolysis, after which it also began to level off. This resulted in powder with distinctive traits as discussed below.

Variation in enzymatic activity between hydrolysis of the synthetic substrate, Azocoll™, and the fish substrate highlights the unique protease-protein interaction that occurs and similar results, using Azocoll™ as a standardising reagent, have been reported elsewhere (Kristinsson and Rasco 2000a).

3.3. Proximate composition

The proximate composition of the freeze-dried powders was determined and compared to that of whole blue whiting (Figure 5.2.). Protein content significantly increased in all the BWSPH powders compared to the starting material (17.23 ± 1.46%; $F(5, 6) = 637.47$, $p < 0.001$).

Protamex hydrolysate had the highest protein content (76.84 ± 0.96%). The protein content of the powder produced with Neutrase 0.8 L had the lowest protein content (40.74 ± 0.18%). A protein yield of below 50% was considered unsuitable for purpose and consequently, this powder was not included in further analyses in this study. Ash content remained similar to starting material for Alcalase, Protamex and Neutrase hydrolysates, with a significant increase and decrease in the Flavourzyme and Savinase BWSPH, respectively. The BWSPH produced with Flavourzyme 500 L was found to contain 19.96 ± 0.05% moisture after freeze-drying. This powder had high moisture sorption behaviour, most likely a result of a large number of available water-binding sites associated with the high degree of hydrolysis and subsequent increased number of lower molecular weight peptides (Figure 5.3.). Previous studies have also found that hydrolysates with high proportions of lower molecular weight peptides have increased moisture sorption behaviour (Hogan and O'Callaghan 2013).

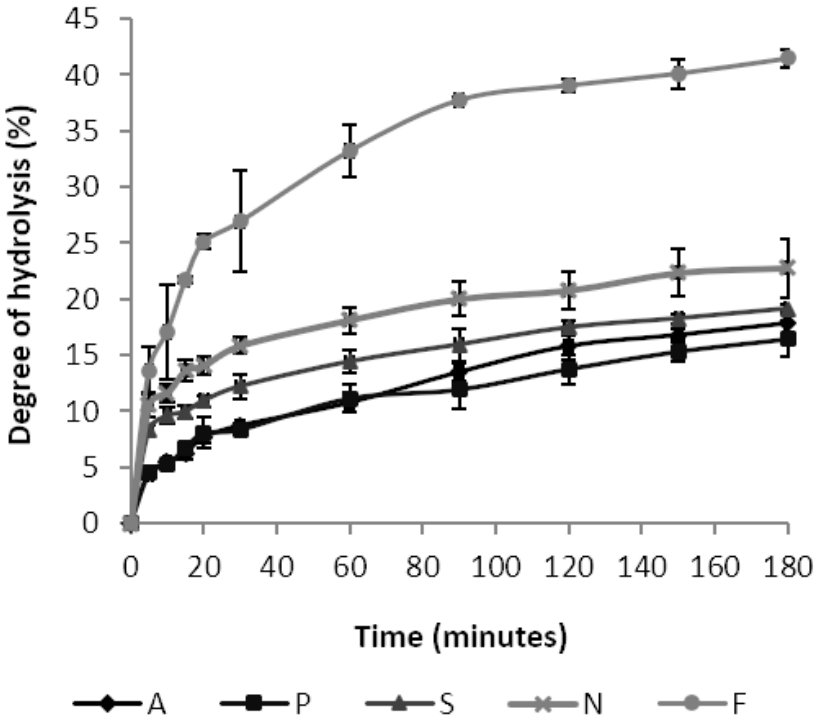

Figure 5.1. Degree of hydrolysis of blue whiting with different commercial proteases (A=Alcalase 2.4L, P=Protamex, S=Savinase 16L, N=Neutrase 0.8L and F=Flavourzyme 500L at optimum pH and temperature.

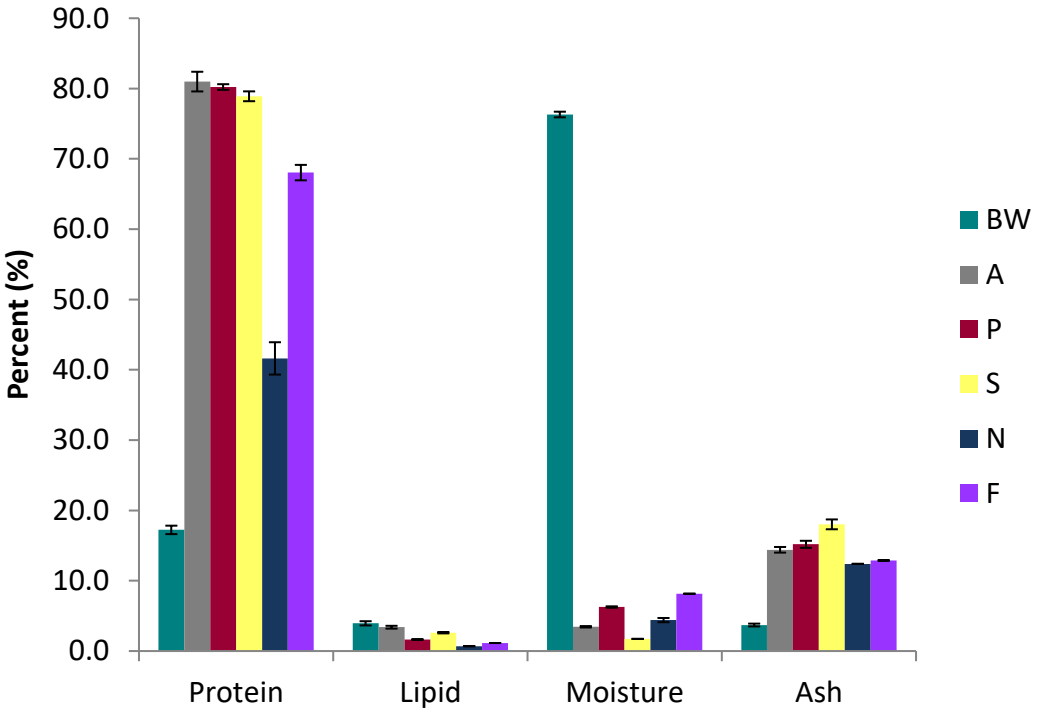

Figure 5.2. The proximate composition of whole blue whiting (BW) and the five BWSPH (A=Alcalase, P=Protamex, S=Savinase, N=Neutrase and F=Flavourzyme).

3.4. Molecular weight distribution profile

The peptide molecular weight distribution of blue whiting prior to enzymatic hydrolysis and the four remaining BWSPH were analysed by high performance liquid chromatography. The blue whiting starting material was composed of 43.20 ± 3.15% peptides > 20 kDa whereas all the BWSPH powders were reduced to smaller peptides with at least 50% of each powder composed of peptides ≤ 0.5 kDa (Figure 5.3.). In agreement with the DH results, Flavourzyme produced the powder with the greatest proportion of small peptides (< 1 kDa). These results suggest that hydrolysis with Flavourzyme yielded a high proportion of shorter (di- and tri-) peptides and free amino acids.

The key characteristics of protein quality for health are digestibility and amino acid composition. The body absorbs dietary proteins in the form of single amino acids and di- and tripeptides. Di- and tripeptides are absorbed more rapidly than that of an equivalent amount of free amino acids (Clemente 2000). Larger intact peptides (≥ tetra-peptides) require additional brush-border hydrolysis to be broken down into their smaller counterparts before absorption (Grimble and Silk 1986). This extra hydrolysis step has an important rate limiting effect on absorption rates. From the molecular weight distribution of peptides in the four BWSPH, it is expected they would all have a fast digestion and absorption rate.

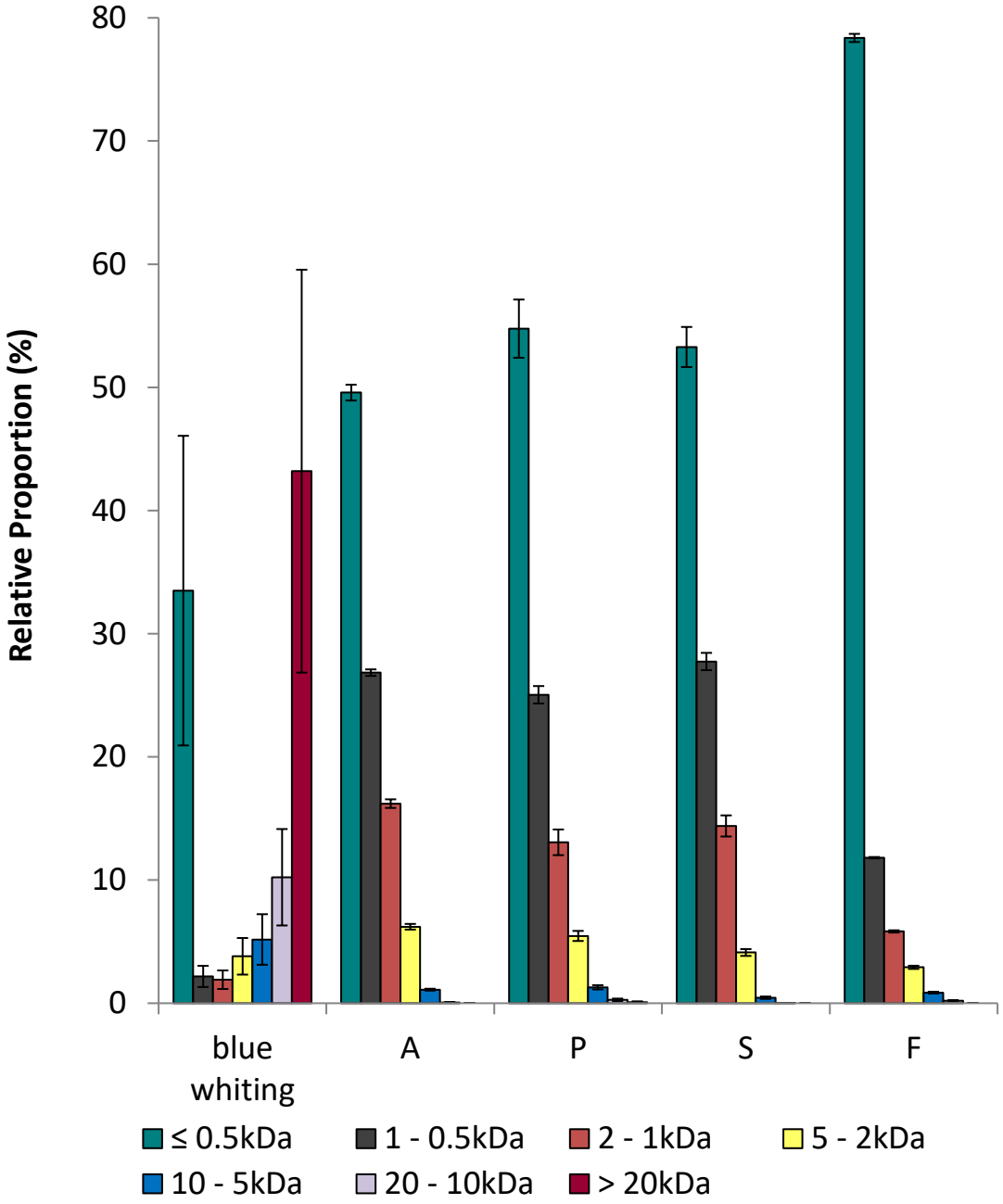

Figure 5.3. Peptide molecular weight distribution profiles of blue whiting prior to enzymatic hydrolysis (blue whiting) and the four BWSPH (A=Alcalase, P=Protamex, S=Savinase, F=Flavourzyme).

3.5. Total and free amino acids

As outlined above, for protein quality a key characteristic to consider is amino acid composition. Therefore, the total and free amino acid compositions of the four BWSPH were determined (Table 5.1.). The three serine proteases; Alcalase 2.4 L, Protamex and Savinase 16 L all produced powders with very similar free amino acid profiles. This is unsurprising as they did not differ significantly in the degree of hydrolysis that they underwent, and the enzymes are all endoproteases with a common reaction mechanism consisting of a catalytic triad of serine, aspartate and histidine. Flavourzyme 500 L includes endoproteases and exopeptidases. It breaks the bonds between hydrophobic amino acids at the N terminal of peptide chains. Thus, the use of this enzyme inevitably results in high numbers of free amino acids.

Of the 20 standard proteinogenic amino acids nine of them are essential amino acids (EAA) which the body cannot produce and therefore need to be acquired in the diet. The UN's WHO has provided a recommended daily allowance of these EAA (Table 5.1.). The four BWSPH produced in this study contain 24.43 to 43.31 g of EAA in 100 g of protein, which equates to 15.90 to 30.85 g of EAA in 100 g of powder. Of the four BWSPH, the Flavourzyme hydrolysate had total EAA close to that recommended by the WHO (Table 5.1.). Within the EAA, the branched chain amino acids (BCAA; valine, leucine and isoleucine), and in particular leucine, stimulate protein synthesis (Kimball and Jefferson 2006). Flavourzyme BWSPH has a slightly lower level of this amino acid compared to the recommended amount, which may moderate protein synthesis, whereas the three other BWSPH provide notably higher amounts of these BCAA which could work to stimulate higher levels of protein synthesis.

Functional food ingredients can be added to products for physicochemical or nutritional benefits. They are ideally bland and colourless, therefore providing minimum impact on the intended sensory characteristics of the food. Since production began, fish hydrolysates have been associated with undesirable sensory properties. The most commonly reported flavours are brothy, fishy and bitter (Nilsang *et al.* 2005). Proteins, and especially free amino acids, can play an important role in flavour (Imm and Lee 1999). Hydrolysis alters the structure of proteins, exposing and releasing interior hydrophobic amino acids. The main amino acids that create a bitter taste are tryptophan, isoleucine, tyrosine, phenylalanine, proline, leucine and valine, in decreasing order (Nilsang *et al.* 2005; Lindsay 2008). These

include the three BCAA that are highly sought after for protein synthesis and the three AA with aromatic rings. The three serine endoproteases produced powders with between 25.74 and 27.17 g $100g^{-1}$ of these bitter amino acids (Table 5.1.), while Flavourzyme BWSPH had slightly fewer (17.52 g 100 g^{-1} protein; Table 5.1.). The amino acids alanine, aspartic acid, serine, methionine and glutamic acid have been associated with umami flavours (Imm and Lee 1999), while threonine, glycine, serine, proline and alanine are important sweet tasting amino acids (Imm and Lee 1999; Shen *et al.* 2012). When considering the total amino acid composition of the BWSPH; umami is likely to be the strongest sensory flavour experienced (Table 5.1.).

Table 5.1. Total and free amino acids (g 100 g^{-1} protein) of the four BWSPH (A=Alcalase, P=Protamex, S=Savinase, F=Flavourzyme) and recommended amino acid requirements of adults (WHO 2007). Branched chain amino acids are highlighted in bold font. WHO=World Health Organisation.

	A	P	S	F	A	P	S	F	WHO
	Free amino acids				Total amino acids				
His	2.86	2.89	4.30	1.48	3.76	3.93	5.14	1.65	1.5
Ile	**0.03**	**0.07**	**0.11**	**2.44**	**4.41**	**3.80**	**4.39**	**2.94**	**3.0**
Leu	**0.10**	**0.14**	**0.13**	**3.70**	**7.59**	**6.73**	**7.51**	**4.48**	**5.9**
Lys	0.00	0.14	0.33	0.22	8.68	8.18	8.57	5.13	4.5
Met	0.22	0.18	0.31	1.98	3.33	2.91	3.41	1.79	1.6
Cys	0.22	0.21	0.19	0.15	1.29	1.10	1.38	0.78	0.6
Met + cys	0.44	0.40	0.50	2.13	4.62	4.01	4.79	2.57	2.2
Phe	1.16	1.11	3.97	2.30	4.06	3.47	4.20	2.24	
Phe + tyr	1.98	2.55	5.42	3.66	5.65	4.42	4.31	3.90	3.0
Thr	0.11	0.13	0.10	3.31	4.41	4.02	4.57	2.75	2.3
Trp	0	0	0	0.91	N/A	N/A	N/A	N/A	0.6
Val	**0.38**	**0.36**	**0.38**	**2.81**	**5.18**	**4.57**	**5.29**	**3.39**	**3.9**
Ala	0.26	0.29	0.29	3.17	6.92	6.50	6.53	4.21	
Arg	0.16	0.19	0.16	2.55	6.44	6.23	6.57	4.07	
Asp	0.14	0.19	0.13	2.13	10.79	10.14	10.47	6.39	
Glu	0.35	0.33	0.27	3.41	16.34	15.61	15.31	9.59	
Gly	0.20	0.17	0.18	1.83	6.56	6.94	6.55	4.36	
Pro	0.10	0.13	0.10	0.92	4.34	4.28	4.25	2.81	
Tau	0.95	1.00	0.96	0.76	0.85	0.85	0.70	0.45	
Tyr	0.82	1.45	1.45	1.36	1.59	0.95	0.11	1.66	
Ser	0.15	0.17	0.16	2.40	4.82	4.52	4.62	2.99	

3.6. Functional properties of BWSPH

Protein hydrolysate functionality is influenced by the amino acid composition and sequence as well as the size of peptides, and the charge and distribution of charges of the molecules. The degree of hydrolysis and percentage of free amino acids are known to significantly alter the functional properties of protein powders (Chalamaiah *et al.* 2013). Functional properties of proteins can be broadly divided into three groups, relating to molecular characteristics; (1.) hydration properties; (2.) protein surface-related properties; and (3.) hydrodynamic/rheological properties (Damodaran 2008). Solubility, emulsification, foaming, viscosity, water binding, gelation, elasticity and flavour/fat binding are all important functions of proteins for the food industry.

3.6.1. Nitrogen solubility

Solubility is often considered the most important physicochemical property for protein hydrolysates, especially when being considered in terms of beverage fortification. Solubility of proteins is influenced by pH, ionic strength, temperature, type of salts, and protein conformation (Damodaran 2008). The food industry demands high solubility across a wide range of pH. In this study, nitrogen solubility in water was found to be above 80% for all BWSPH across the pH range 2−10. Similar levels of solubility have been recorded for other fish protein hydrolysates (Klompong *et al.* 2007). Taking the four BWSPH powders together, a significant difference in solubility between the pH levels 2 and 4 was found (87.5% and 91.1%; $F(4, 19) = 3.649$, $p < 0.05$; Figure 5.4a.). Comparing each of the BWSPH powders across all pH values, Alcalase hydrolysates were the most soluble, significantly more so than Protamex or Flavourzyme hydrolysates ($F(3, 19) = 83.212$, $p < 0.001$). Protein solubility is the thermodynamic manifestation of the equilibrium between protein–protein and protein–water interactions. The amino acids present and their sequences within peptides largely determine moisture-absorption and water dynamics (Yang *et al.* 2017). The reduced solubility of Flavourzyme hydrolysate was a predictable result of the higher water content of this powder. A greater proportion of small peptides and free amino acids were present which, due to their hydrophilic nature, were bound to water molecules before the nitrogen solubility analysis. However, all the BWSPH had high solubility which would assist the production of an attractive appearance and smooth mouth-feel to any food or beverage product. For beverage

fortification, good solubility at low pH ranges is crucial. Carbonated and fruit drinks tend to be acidic by nature. At low pH, the charge on the weakly acidic and basic side chains of amino acids are influenced and become less soluble with possible precipitation (Gbogouri *et al.* 2004). The results of the solubility test showed that all the BWSPH had excellent solubility at low pH (2 and 4). A beverage matrix will, however, have other compounds in it which may promote or impede solubility. The vitamin-tea beverage in which the BWSPH were dissolved had a pH of 3.3. All the BWSPH displayed excellent solubility within the beverage (> 85%) and there was no significant difference in solubility between the powders ($F(3, 4) = 11.6$, $p = 0.019$; Figure 5.4b.).

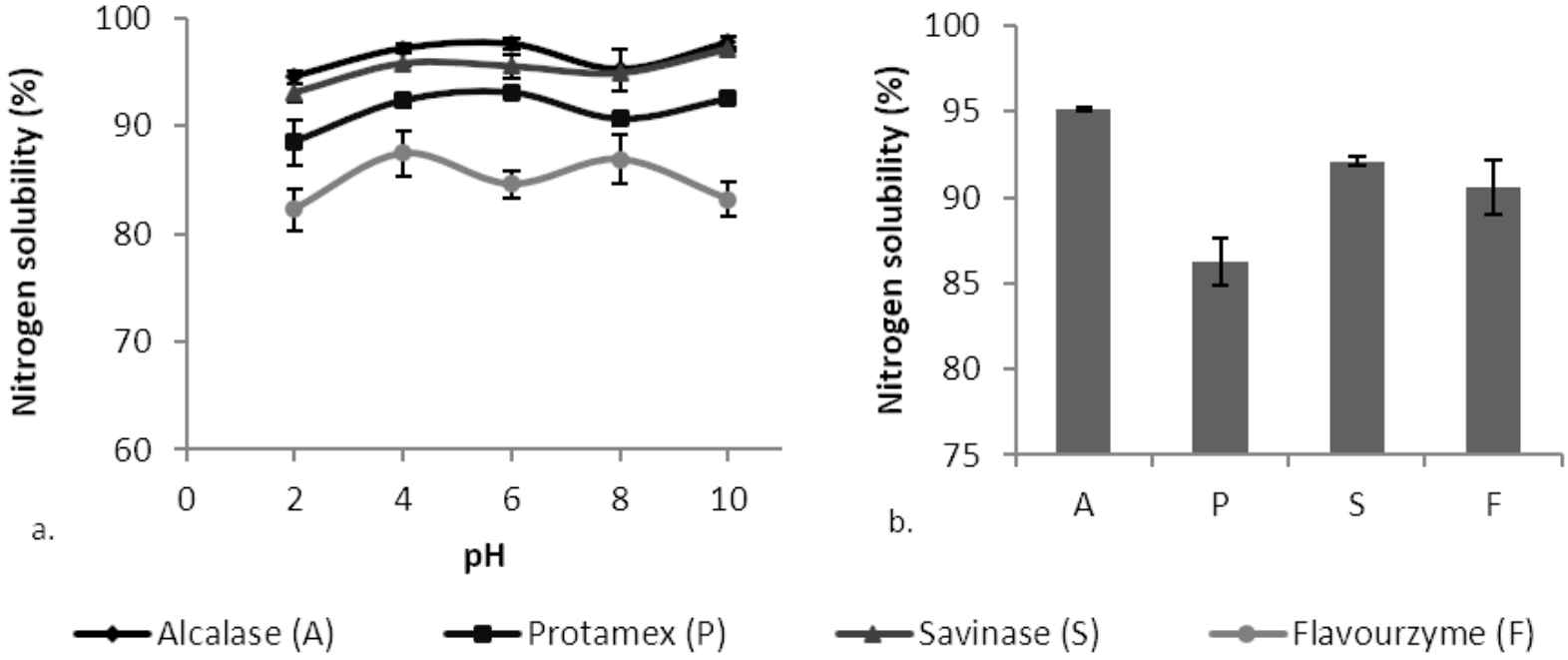

Figure 5.4. Nitrogen solubility (%) of the four BWSPH in; water at different pH levels (2, 4, 6, 8, 10) (a.) and, a vitamin-tea beverage (b.).

3.6.2. Emulsifying properties

EAI (m^2 g^{-1}) and ESI (min) of the BWSPH at different concentrations of powder (0.5%, 1%, 2% w/v) are shown in Table 5.2. All the BWSPH had reduced EAI as their concentration increased. The difference among the BWSPH was most significant at the 0.5% (w/v) concentration, with Flavourzyme (70.25 ± 1.76 m^2 g^{-1}) having the highest EAI and Alcalase (37.81 ± 0.68 m^2 g^{-1}) having the lowest ($F(1, 12) = 826.30$, $p < 0.001$). The ESI differed significantly depending on concentration as well as between BWSPH. Similar to the EAI, the difference in ESI between BWSPH was largest at concentration of 0.5% (w/v) ($F(3, 24) = 213.06$, $p < 0.001$). Protamex hydrolysate had the highest ESI, recorded at 1% and 2% (w/v) (7.82 ± 0.17 min; 7.57 ± 0.47 min, respectively). Emulsification occurs during homogenisation when proteins are absorbed to the surface of oil droplets as they are formed, creating a membrane which prevents them from consolidating (Sila *et al.* 2014). In low concentration solutions, proteins adsorb at the oil/ water interface by diffusion, whereas, at high concentrations the activation energy barrier prevents diffusion and the proteins tend to accumulate in the aqueous phase (Thiansilakul *et al.* 2007). In line with this, the results of this study found that higher concentrations of protein prevented as many oil droplets from being preserved.

3.6.3. Oil binding capacity

Oil binding occurs through physical entrapment. For this reason, the higher the bulk density of the protein the greater the OBC (Tanuja *et al.* 2012). The OBC of all four BWSPH was low, though Savinase had a statistically higher value compared to the other three powders ($F(3, 8) = 17.73$, $p < 0.05$; Table 5.2.). Other studies investigating the functional properties of fish protein hydrolysates have reported higher OBC than those found here (Geirsdottir *et al.* 2011; Jemil *et al.* 2014). The OBC values reported in this study are comparable to those reported for soy protein (Geirsdottir *et al.* 2011). The oil binding capacity of proteins will affect functional characteristics as well as taste of the final product. It is considered an important attribute for proteins that are to be used in the meat and confectionery industries.

Table 5.2. Emulsifying activity index (EAI, $m^2 g^{-1}$) and emulsion stability index (ESI, min) at different w/v (0.5%, 1%, 2%), and oil binding capacity (OBC, $g g^{-1}$) of the BWSPH.

	EAI (m^2g^{-1})			ESI (min)			OBC ($g g^{-1}$)
BWSPH conc.	0.5%	1%	2%	0.5%	1%	2%	
Alcalase	37.81 ± 0.68	17.41 ± 0.81	18.44 ± 1.69	1.95 ± 0.76	3.57 ± 0.51	4.83 ± 0.73	1.45 ± 0.01
Protamex	57.73 ± 0.35	21.37 ± 2.13	18.86 ± 0.71	2.97 ± 0.56	7.82 ± 0.17	7.57 ± 0.47	1.44 ± 0.03
Savinase	58.55 ± 0.70	34.73 ± 1.09	24.29 ± 2.43	3.51 ± 0.43	5.42 ± 0.15	4.89 ± 0.23	1.56 ± 0.01
Flavourzyme	70.25 ± 1.76	22.69 ± 0.52	19.53 ± 0.56	5.65 ± 0.32	5.10 ± 0.82	7.01 ± 0.11	1.36 ± 0.01

3.6.4. Colour

Colour is an important part of the sensory experience of food and beverage consumption, contributing to the quality and appeal of product to the consumer. The colour of fish protein hydrolysates will depend on the composition of raw material, enzymes used and the conditions of hydrolysis. Colour analysis of the four BWSPH in solution was performed at concentrations of 1% and 10% (w/v). Colour is reported in terms of lightness (L∗), red-green colour (a∗) and yellow-blue colour (b∗) as described by the Lighting International Commission. There were statistically significant differences between the colours of specific BWSPH on all axes at the concentration of 10% (w/v). However, once diluted to 1% (w/v), the only statistically significant difference that remained was on the yellow-blue axis. Superficially, all the solutions were light yellow in colour at both concentrations. On analysis, Protamex (49.05 ± 0.36) was significantly lighter than Alcalase, Savinase and Flavourzyme hydrolysates at a concentration of 10% (w/v) ($F(4, 10) = 57.12$, $p < 0.01$). At the same concentration, on the green – red spectrum, Flavourzyme hydrolysate was on the red side of neutral which was significantly different to the other BWSPH, which were all on the green side of neutral ($F(4, 10) = 110.55$, $p < 0.01$). Finally, Flavourzyme hydrolysate (11.72 ± 2.69) was significantly more yellow than the BWSPH, apart from Protamex hydrolysate at a concentration of 10% (w/v) ($F(4, 10) = 19.47$, $p < 0.01$). These results are in line with other studies that have shown that increased hydrolysis causes darkening through enzymatic browning (Jemil *et al.* 2014).

3.6.5. Antioxidant properties of BWSPH

Antioxidants are important in health as well as food preservation. Food and pharmaceutical industries often use synthetic antioxidants in their products, however; there is an interest in finding safe and potent natural antioxidants. In this study, we compared the antioxidant activity of the BWSPH using three different assays; DPPH free radical scavenging, reducing power assay and ferrous chelating ability. Using the DPPH assay, significant differences in the scavenging ability of all BWSPH were found ($F(3, 48) = 838.37$, $p < 0.001$).

Differences in scavenging ability became significant at concentrations of 3.0 mg mL^{-1} and above, at which Flavourzyme hydrolysate had a significantly greater scavenging ability compared to the other BWSPH ($p < 0.001$) until at 18 mg mL^{-1}, where Alcalase hydrolysate also displayed 100% scavenging ability (Figure 5.5a.). Protamex hydrolysate had significantly

higher reducing power compared to the other BWSPH ($F(4, 60) = 909.4$, $p < 0.001$; Figure 5.5b.). Flavourzyme hydrolysate had the second highest reducing power, while Alcalase and Savinase hydrolysates were significantly lower ($F(4, 60) = 909.4$, $p < 0.001$). These three BWSPH showed a linear increase in reducing power, while Protamex hydrolysate levelled off after a concentration of 6 mg mL^{-1} (Figure 5.5b.). Finally, in the ferrous chelating ability assay, the antioxidant ability of all the BWSPH increased in a similar concentration dependent manner. However, statistically significant differences in chelating ability were found between all BWSPH except for Flavourzyme and Alcalase hydrolysates ($F(3, 48) = 51.29$, $p < 0.001$). When the BWSPH were tested at the highest concentration (18 mg mL^{-1}), they all displayed similar ferrous chelating ability (Figure 5.5c.).

Different mechanisms of oxidation occur in the three assays, as outlined elsewhere (Thiansilakul *et al.* 2007). Therefore, it is understandable that antioxidant ability varied in the BWSPH between assays. Taking the three assays together, however, Flavourzyme hydrolysate appeared to have the greatest overall antioxidant ability. Previous studies have reported that protein hydrolysates with a higher DH and lower molecular weight peptides interact more effectively with radicals to interfere with the oxidation process (Klompong *et al.* 2007).

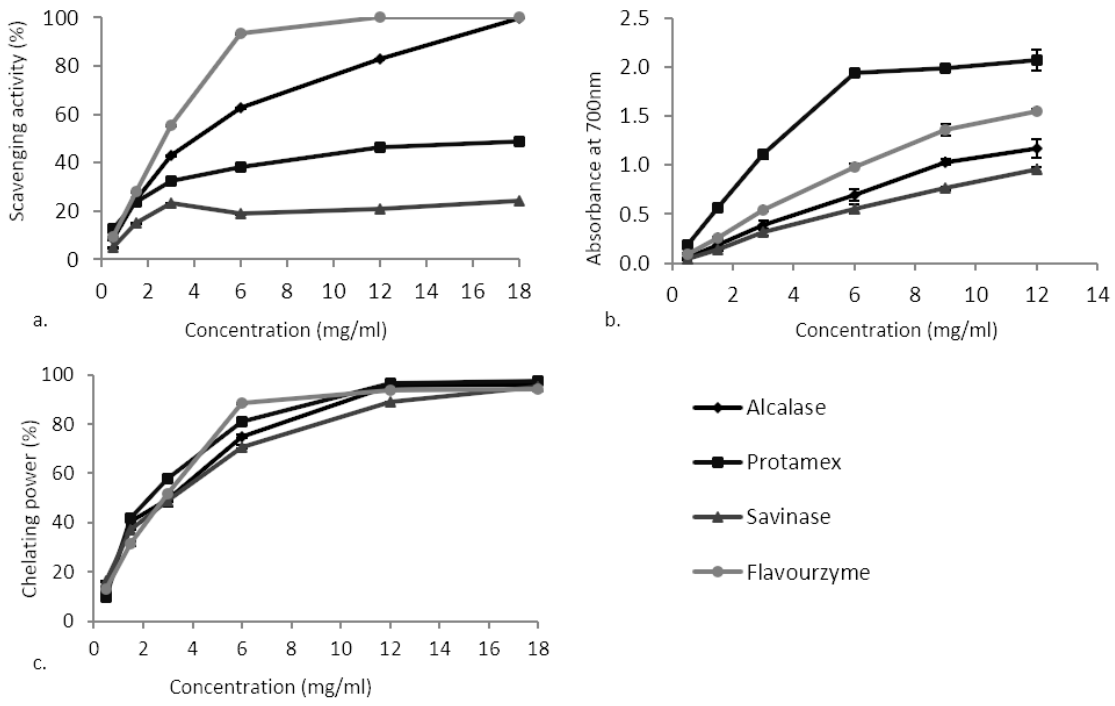

Figure 5.5. Antioxidant effect of the four BWSPH (Alcalase, Protamex, Savinase and Flavourzyme) assessed using three antioxidant assays; DPPH free radical scavenging (a.), reducing power (b.), and ferrous chelating (c.).

4. CONCLUSIONS

This study has shown that whole blue whiting fish are a suitable source of highly nutritious protein. Through hydrolysis, the use of commercial enzymes allowed for the extraction and alteration of protein from a low value source to produce highly digestible, low molecular weight peptide powders that have EAA content close to that required for human protein synthesis. Producing powders with a high DH (> 15%) allowed for high solubility but concurrently reduced other functional properties. All the powders showed antioxidant properties which will provide benefits for food preservation as well as an extra dimension to a health ingredient. Flavourzyme 500 L produced product with significantly more free amino acids which potentially provide properties that are less desirable in terms of absorption rates but may improve flavour. The BWSPH had mild colouring and blended well with a commercial test beverage. Further research will aim to test consumer acceptability of the supplemented beverages with a sensory panel and investigate shelf life characteristics.

Acknowledgements

This work was supported by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. [grant number EPSPG/2015/57]. This work was also supported in part by The APC Microbiome Institute, which is funded by Science Foundation Ireland (Grant Number SFI/12/RC/2273).

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Chapter 6

Replacing fishmeal with plant protein in Atlantic salmon (*Salmo salar*) diets by supplementation with fish protein hydrolysate

Published: *Scientific Reports* (2020)

ABSTRACT

The effects of feeding an 80% plant protein diet, with and without fish protein hydrolysate (FPH) supplementation, on the growth and gut health of Atlantic salmon (*Salmo salar*) were investigated. Fish were fed either (a.) a control diet containing 35% fishmeal, (b.) an 80% plant protein diet with 15% fishmeal, (c.) an 80% plant protein diet with 5% fishmeal and 10% partly hydrolysed protein, or (d.) an 80% plant protein diet with 5% fishmeal and 10% soluble protein hydrolysate. Fish on the 80% plant, 15% fishmeal diet were significantly smaller than fish in the other dietary groups ($p < 0.001$). However, partly-hydrolysed protein supplementation allowed fish to grow as well as fish fed the control fishmeal diet ($p > 0.05$). Fish on the FPH diets had significantly higher levels of amino acids in their blood, including 48% and 27% more branched chain amino acids compared to fish on the 35% fishmeal diet, respectively. Plant protein significantly altered gut microbial composition, significantly decreasing α-diversity. Spirochaetes and the families *Moritellaceae, Psychromonadaceae, Helicobacteraceae* and *Bacteroidaceae* were all found at significantly lower abundances in the groups fed 80% plant protein diets compared to the control fishmeal diet.

1. INTRODUCTION

Fishmeal is generally considered the gold standard dietary protein source for many fish species. Its production is based upon wild marine fish of no commercial value (Hertrampf and Piedad-Pascual 2012). However, today it is considered both environmentally and ecologically unsustainable and there are societal and economic pressures on the aquaculture industry to find alternative proteins. Removing fishmeal from the diets of herbivorous and omnivorous species has been readily achieved, but this has been more difficult to implement in carnivorous fish and crustaceans (Turchini *et al.* 2019). It has been generally found that up to 50% fishmeal protein can be replaced by plant proteins in carnivorous fish diets without any negative effects on growth or fish welfare issues (Hardy 2010). Plant proteins are the most common replacements for fishmeal in aquafeeds. They are cost-effective but also preferred because of the negative consumer perception around the use of terrestrial animal by-products (e.g. b*ovine spongiform encephalopathy* prion disease) for feeding fish (Collins *et al.* 2013; Gajardo *et al.* 2017; Shepherd *et al.* 2017). However, when aquafeeds high in plant protein (> 50%) are formulated to provide the required balance of amino acids and other essential nutrients (e.g. fatty acids, macro and trace metals), the growth performance obtained is inferior to that of fish fed fishmeal-based diets (Collins *et al.* 2013; Gajardo *et al.* 2017; Turchini *et al.* 2019). These shortcomings are often the result of plant proteins possessing anti-nutritional factors (e.g. phytate, saponins, lectins) and indigestible carbohydrates, as well as less efficient protein digestion and amino acid absorption (Lall and Anderson 2005).

There has been considerable research on re-formulating aquafeeds using novel ingredients and nutritional supplements (e.g. exogenous enzymes, bioactive compounds and bioavailable trace metals) that complement plant proteins and help to meet the needs of aquaculture species. Creating sustainable feeds that promote fish welfare, maximise growth potential while remaining cost efficient is a prominent challenge for the aquaculture industry. Some promising work is emerging to suggest that diets with very low or no fishmeal will be possible with careful formulation in the future (Espe *et al.* 2006; Kousoulaki *et al.* 2012). However, to date, it has been suggested that, for optimal growth, a minimum of 5% fishmeal is required to provide unidentified growth factors, thought to be naturally occurring trace and ultra-trace compounds such as amines and steroids (Espe *et al.* 2007; Hardy 2010).

Fish protein hydrolysates (FPH) are products from either chemical (e.g. acid and alkaline) or enzymatic (e.g. protease) breakdown of fish proteins into single amino acids, peptides and oligopeptides. High quality FPH can be produced from fish processing by-products, fishery by-catch, and low-value pelagic species not currently directly consumed by humans. They are considered a suitable source of protein for human and animal nutrition because of their balanced amino acid composition and their low molecular weight, allowing higher gut absorption rates (Benjakul *et al.* 2014; Egerton *et al.* 2018). Their addition at low concentrations (18 – 24%) have been found to significantly increase individual specific growth rates of adult Atlantic salmon (Hevrøy *et al.* 2005). More recently, Atlantic salmon, at the fast-growing seawater stage, were found to grow equally well on a diet consisting of plant proteins supplemented with a combination of 5% fishmeal, 5% fish soluble protein and 3% squid hydrolysate as on a fishmeal control diet (Espe *et al.* 2007). The supplemented fish-derived fractions of this diet increased palatability and provided sufficient bioavailable nutrients to compensate for the nutritional shortcomings of the plant protein ingredients (e.g. antinutrients and lower bioavailability of nutrients).

FPH, added at appropriate levels, has been reported to increase survival and growth rates, decrease malformation rates, increase enzyme activity, modify nutrient transport patterns in the intestine, improve nutrient absorption and induce non-specific immune responses in larvae, fry and adult fish (Bøgwald *et al.* 1996; Cahu *et al.* 1999; Espe *et al.* 1999; Murray *et al.* 2003; Aksnes *et al.* 2006; Liang *et al.* 2006). FPH added to Atlantic salmon diets have resulted in positive immune modulation (enhanced levels of superoxide anion production in head kidney leucocytes) in adult fish (Bøgwald *et al.* 1996) and increased feed intake (12.5% greater than control) and growth (1.8% higher specific growth rate compared to control) in post-smolts (Hevrøy *et al.* 2005).

In this study, we investigated the effects of high plant-protein/ low fishmeal diets, with and without FPH supplementation on growth performance and gut health in Atlantic salmon parr, on-grown in freshwater. Only two previous studies conducted in the past 25 years investigated effects of FPH supplementation on growth performance in juvenile freshwater Atlantic salmon, neither of which used plant protein-based diets (Gildberg *et al.* 1995; Berge and Storebakken 1996). Freshwater juvenile Atlantic salmon have much higher growth rates compared to seawater, post-smolt salmon (Rollin *et al.* 2003). Furthermore, it is important for juveniles to have a high nutritional status and energy turnover in order to successfully

undergo smoltification and a generally positive relationship between salmon smolt size and survival is frequently noted (Sissener *et al.* 2009; Beckman *et al.* 2017). Thus, there is a high requirement for aquafeeds, focussed towards this life stage, which provide optimal growth and fish health while meeting market demands in sustainability. This study addresses this area of research. In a 12-week feeding trial, four different diets that were iso-nitrogenous and iso-lipidic, differing only in protein source, were fed to Atlantic salmon parr and subsequent growth and gut health were investigated.

2. MATERIALS AND METHODS

2.1. Experimental diets

The tested fish protein hydrolysates (Biomarine Ingredients Ireland Ltd., Monaghan, Ireland) were produced from whole blue whiting (*Micromesistius poutassou*) frozen directly after catching from the wild. Following thawing, lipids and bone were removed from the blue whiting and the remaining raw material was enzymatically hydrolysed. The water-soluble protein hydrolysate fraction and the insoluble partly hydrolysed protein fraction were separated and spray-dried to prevent thermal damage to the protein. The SPH hydrolysate was composed of lower molecular weight peptides and single amino acids and contained 91% protein. The PHP hydrolysate was composed of low and medium molecular weight peptides and contained 68% protein.

Four diets were formulated and manufactured by the Aquaculture Nutrition and Aquafeed Research Unit (ANARU) at Carna Research Station, Ryan Institute, National University Ireland Galway. Commercially available feed ingredients were used, and diets were formulated to meet the dietary requirements of appropriately sized salmon, including vitamin and mineral requirements (NRC 2011). Formulation and proximate composition of experimental diets are shown in Table 6.1. Diet 1 (FM), the control diet, was formulated to replicate fishmeal-based commercial salmon aquafeeds, containing 35% fishmeal. The other three diets were high in plant protein (80%) and low in fish-derived protein. Diet 2 contained 15% fishmeal (PL), diet 3 contained 5% fishmeal and 10% partly hydrolysed fish protein (PHP) and diet 4 contained 5% fishmeal and 10% soluble protein hydrolysate (SPH). All diets were iso-nitrogenous and iso-lipidic content (Table 6.1.). The total amino acid content of the four diets and the PHP and SPH supplement are outlined in Table 6.2. The experimental diets were extruded (1 and 2mm pellets) in a single screw extruder (PM-80, Bottene, Vicenza, Italy) and dried at 40 °C in a dehumidifying oven. There were no differences in physical quality or sinking properties of the four diets.

Table 6.1. Treatment diets formulation and proximate composition (%, dry matter), and estimated costs of variable protein ingredients (€/ metric tonnes (MT)).

Diet Formulation	**FM**	**PL**	**PHP**	**SPH**
Fishmeal [a]	35.00	15.00	5.00	5.00
Soy meal concentrate [b]	14.34	40.33	40.16	35.68
PHP [c]	-	-	10.00	-
SPH [c]	-	-	-	10.00
Fish oil [a]	14.00	14.00	14.00	14.00
Wheat gluten [d]	9.00	9.00	9.00	9.00
Pea protein [d]	9.00	9.00	9.00	9.00
Rape seed oil [e]	1.18	3.29	3.14	4.29
Potato starch [f]	13.98	5.88	6.20	9.53
Vitamin & Mineral Premix [g]	2.00	2.00	2.00	2.00
Lysine [h]	0.50	0.50	0.50	0.50
Antioxidants [i]	0.50	0.50	0.50	0.50
Methionine [h]	0.30	0.30	0.30	0.30
Molasses [j]	0.20	0.20	0.20	0.20
Proximate Composition				
Moisture	5.54	5.25	5.8	5.91
Crude Protein	47.78	47.67	47.73	46.46
Crude Lipid	19.22	19.46	19.29	19.97
Ash	8.02	5.68	4.64	4.86
Cost Comparison (€/ MT Feed)				
Fishmeal [k]	456.17	195.50	65.17	65.17
Soy meal concentrate [l]	84.41	237.40	236.40	210.03
PHP [c]	-	-	240.00	-
SPH [c]	-	-	-	400.00
Total	540.58	432.90	541.56	675.19

[a] United Fish Industries, Grimsby, UK.
[b] HP100, Hamlet proteins, Horsens, Denmark
[c] Biomarine Ingredients Ireland, Monaghan, Ireland.
[d] Roquette Freres, Lestrem, France.
[e] KTC Edibles Ltd., Wednesbury, UK.
[f] Terros, Origny-Sainte-Benoite, France.
[g] Premier Nutrition Products Ltd., UK. Manufacturer analysis: Ca 12.09%, Ash 78.71%, Na 8.86%, Vitamin A 1.0µg/kg, Vitamin D 0.10%, Vitamin E 7.0g/kg, Cu 250mg/kg and P 5.2g/kg.
[h] Biomar Ltd., Northshore Road, Grangemouth Docks, Scotland.
[i] Barox plus liquid, Kemin Europa N.V., Belgium.
[j] Target Baits, Whitchurch, Shropshire, UK.
[k] Indexmundi.com: World Bank, March 2019.
[l] Alibaba.com: Agrosul Agroavicola Industrial SA, Brazil, April 2019.

Table 6.2. Total amino acid composition of the two supplementary protein ingredients (% of powder), the four treatment feeds and the essential amino acid requirements of Atlantic salmon (NRC 2011) (% of diet dry matter; ND, not determined).

	Supplements		**Diet**				**Atlantic salmon**
	PHP	**SPH**	**FM**	**PL**	**PHP**	**SPH**	**(av. 0.2 - 500g)**
Arginine	4.63	5.71	3.03	3.18	3.11	3.15	1.81
Histidine	1.62	1.17	1.41	1.39	1.26	1.38	0.80
Isoleucine	3.66	3.01	2.12	2.14	2.16	2.02	1.32
Leucine	5.91	5.63	3.75	3.79	3.73	3.65	2.31
Lysine	6.48	8.51	3.57	3.16	3.10	3.25	2.55
Methionine + Cystine	3.14	2.67	2.08	1.75	1.81	2.04	1.29
Phenylalanine + Tyrosine	6.76	3.92	3.63	3.90	3.86	3.56	2.70
Threonine	3.63	3.92	1.88	1.85	1.81	1.75	1.58
Tryptophan	ND	ND	ND	ND	ND	ND	0.36
Valine	4.12	4.05	2.34	2.39	2.37	2.26	1.77
Cystic acid	ND	ND	0.87	0.94	1.10	0.99	
Taurine	ND	ND	0.39	0.19	0.27	0.32	
Asparagine	7.55	8.28	4.58	4.71	4.88	4.77	
Serine	3.66	4.11	2.20	2.23	2.35	2.26	
Glutamic acid	9.59	15.00	9.39	9.42	10.09	9.63	
Glycine	3.75	6.51	2.61	2.06	2.24	2.29	
Alanine	4.54	5.93	2.42	2.04	2.12	2.18	
Proline	2.84	3.70	2.61	2.54	2.77	2.60	

2.2. Fish and rearing conditions

The experiment was carried out at Salmon Springs Ltd., freshwater juvenile salmon rearing facility in Co. Galway, Ireland. Atlantic salmon were raised from eggs (donated kindly by Stofnfiskur, Iceland) on site. Prior to the experiment, the fish were fed commercial diets (Skretting UK, Cheshire, UK). At the start of the experiment, salmon (8.44 ± 0.78 g) were randomly distributed into 1 m^3 fibreglass tanks (at a density of 6.5 kg/m^3 in 0.4 m^3 of water, *n* = 3). The tanks were set up on a flow-through system and supplied with natural spring freshwater at a flow rate of 4 L min^{-1} and further aerated with air pumps. A natural photoperiod was used which ranged from 14.75 – 16.75 h daylight. The average water temperature during the trial was 11.2 ± 0.6 °C and dissolved oxygen level was recorded throughout (9.1 ± 0.71 mg L^{-1}). Triplicate groups of fish were fed one of the four treatment diets via automatic feeders during daylight hours (~1.5% BW) for 12 weeks. Tank weights were measured fortnightly to allow for feed adjustments. Feeding was withheld 24 hr prior to morphometric measurements to ensure that fish were clear from residue feed. All experiments were approved by the Ethics Committee of University College Cork, licenced by HPRA, Ireland (project authorisation AE19131/P068) and in full accordance with the European Community Council Directive (2010/EU/63).

2.3. Sample collection and analyses

2.3.1. Sample collection

Fish were sampled the day before the dietary treatment began and at the end of the trial. Fish were not starved in advance of sampling to ensure collection of intestinal contents and reduce possible alterations of blood amino acid concentrations. From each tank, eight random fish were sampled, euthanised and blood was taken immediately from the caudal vein with a heparinised 25G needle and 1 mL syringe. Blood samples and carcasses were stored at -20 °C until analysis.

A further eight fish per tank were culled and dissected to collect organs. The gastrointestinal tract from the stomach to the anus was removed from the peritoneal cavity, placed on a sterile petri dish and aseptically dissected. The intestinal contents from the distal intestines were frozen immediately on dry ice before transferring to -80 °C storage for later analysis. Livers were removed and weighed.

At the end of the trial, after last sampling, remaining fish were starved for 24 h. Subsequently, wet weight and forktail length of each individual fish from all tanks were measured.

2.3.2. Proximate composition and amino acid determination

Feed ingredients, test diets and fish body proximate composition were determined using AOAC (2002) methods. Whole carcasses from each tank were pooled in duplicate and homogenised (Robot Coupe blixer 2 commercial food processor, Stephens Catering Equipment Co. Ltd., Ireland) to create a uniform mince. Moisture content was determined gravimetrically after drying at 105 °C for 24 h. Ash content was measured by incinerating the samples at 550 °C for 16 h. Total nitrogen content was determined by the Kjeldahl method and crude protein content was estimated by multiplying total nitrogen content with 6.25 conversion factor. Lipid content was determined gravimetrically after extraction following a modified Bligh and Dyer method (Smedes 1999).

Whole blood samples were used for the analysis of free amino acid content according to the methods of McDermott *et al.* (2016). Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol UK Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high-performance cation exchange column. For total amino acid content of feeds, proteins were hydrolysed in 6 N HCl at 110 °C for 23 h and the resulting hydrolysates analysed as per free amino acid method.

2.3.3. Intestinal morphology

At the end of the feeding trial, three fish were sampled from each tank (n = 9) and the posterior intestine was fixed in 10% neutral buffer formalin for histological processing. Samples were subsequently dehydrated and embedded into wax for sectioning. Sample sections were stained using Mayer's Haematoxylin and Eosin (Thermo Fisher Scientific, Waltham, Massachusetts, US). Measurements of the gut were carried out using a light microscope and processed using ImageJ (Schneider *et al.* 2012).

2.3.4. Gut microbiota 16S rRNA sequencing

DNA extraction from samples was performed using a QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen Ltd, Manchester, England) according to the manufacturer's protocol with the following modifications outlined by Dehler *et al.* (2017). Extracted DNA was quantified by NanoDrop™ spectrometry (Thermo Fisher Scientific, Waltham, Massachusetts, US). The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S metagenomic sequencing library protocol. The PCR reactions were performed in a 25 µL reaction volume containing DNA template, 12.5 µL Biomix Red (Bioline, Memphis, USA), 5 µL each of forward and reverse primers (1 µM), and PCR grade water to final volume. PCR amplification conditions included initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 5 min. PCR products were cleaned using AMPure XP magnetic bead-based purification (Beckman Coulter Life Sciences Brea, California, United States). This was followed by indexing PCR, which attached Nextera XT barcodes and Illumina® sequencing adapters to the 5' overhangs and another round of AMPure XP clean-up. After quantifying the samples, using Invitrogen Qubit 4 Fluorometer and high sensitivity DNA quantification assay kit (Thermo Fisher Scientific, Waltham, Massachusetts, US), they were pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. Samples were sequenced on the MiSeq sequencing platform at the Teagasc Sequencing Facility, using a 2 x 300 bp cycle kit, following standard Illumina® sequencing protocols.

2.3.5. Bioinformatics analysis

Three hundred base pair paired-end reads were assembled using FLASH. The QIIME suite of tools, v1.8.0, was used for further processing of paired-end reads, including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds (Caporaso *et al.* 2010). De-noising, chimera detection and operational taxonomic unit (OTU) grouping at 98% similarity were performed using USEARCH v7 (64-bit) (Edgar 2010). Taxonomic ranks were assigned by the alignment of OTUs using PyNAST to the SILVA SSURef database release 128 (Quast *et al.* 2012). Alpha and beta diversities were calculated using QIIME on weighted Unifrac distance matrices.

16S microbiota data was entered into Calypso (Zakrzewski *et al.* 2016) for further analysis and statistical testing. Principal co-ordinate analysis (PCoA) plots were visualised using Bray-Curtis calculated distances and differences between dietary treatments were determined using permutational multivariate analysis of variance (PERMANOVA-Adonis). The Benjamini-Hochberg adjustment procedure was applied with the false discovery rate (FDR) set at 20% to correct for multiple testing.

2.4. Calculations and statistics

Specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and hepatosomatic Index (HSI) were expressed as the following:

$$SGR\ (\%) = \frac{lnW_2 - lnW_1}{Feeding\ days} \times 100 \qquad \text{Eq. 6.1.}$$

$$FCR = \frac{Feed\ consumed/fish}{W_2 - W_1} \qquad \text{Eq. 6.2.}$$

$$PER = \frac{W_2 - W_1}{Protein\ consumed/fish} \qquad \text{Eq. 6.3.}$$

$$HSI\ (\%) = \frac{W_l}{W_f} \times 100 \qquad \text{Eq. 6.4.}$$

Where, W_1 and W_2 are average initial and final fish weights, respectively, W_l is the weight of liver and W_f is the weight of fish at the time of sampling and ln is the natural logarithm.

Statistical analyses were performed using Microsoft Excel® and the SPSS® computer programs (SPSS Statistical Software, Inc., Chicago, IL.). All data were subjected to Analysis of Variance (ANOVA), or non-parametric alternative where appropriate, and pairwise comparisons were conducted by Tukey's test. The significance level was determined at the 95% probability level ($p < 0.05$). GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used for the generation of figures.

A cost comparison of the diets was made by comparing the cost of the variable protein ingredients; fishmeal, soy protein concentrate (SPC) and the experimental protein

supplement hydrolysates; PHP and SPH. The value for the fishmeal was taken from the website indexmundi.com, a website providing detailed country statistics, charts, and maps compiled from multiple sources. The source reported was the World Bank. The cost for SPC was taken from the website Alibaba.com, a global wholesale commodities website.

3. RESULTS

3.1. Growth performance, feed intake and biometric measurements

This study consisted of a 12-week dietary intervention with Atlantic salmon parr to investigate the effects of alternative protein sources on the growth and gut health of the fish. Fish were fed either (a.) a control diet containing 35% fishmeal (FM), (b.) an 80% plant protein diet with 15% fishmeal (PL), (c.) an ~80% plant protein diet with 5% fishmeal and 10% partly hydrolysed fish (PHP), or (d.) an ~80% plant protein diet with 5% fishmeal and 10% soluble protein hydolysate (SPH; Table 6.1.). Salmon parr were placed in triplicate tanks at an initial density of 16.2 kg L^{-1} (average weight 8.44 ± 0.78 g, F (11, 1188) = 1.567, p = 0.103). Average tank density for all treatment groups had reached 20 kg L^{-1} by day 40 of the feeding trial and tanks were maintained at this density for the remainder of the trial. Feed utilisation and conversion measures were calculated from tank averages (Table 6.3.). Feed intake was similar for all tanks and ranged from 24.81 – 27.43 g $fish^{-1}$. Apparent digestibility coefficient of dry matter was greatest for FM and SPH groups (80.83 and 80.00%) and notably lower in fish fed the PL diet (70.77%). Specific growth rates were highest for FM and PHP fish and lowest for PL fish. Similarly, PHP and FM fish showed the highest protein efficiency ratio, while SPH fish had the lowest protein efficiency ratio. Conversely, feed conversion ratio was lowest for PHP and followed by FM fish. Although these differences were observed, they were not found to be statistically significant (p = 0.189). Hepatosomatic index is the ratio of liver weight to body weight. It provides an indication on the status of energy reserve in the fish. Hepatosomatic index was significantly higher in the PHP fish compared to PL fed fish (F (3, 8) = 4.469, $p < 0.05$; Table 6.3.).

The principle fish growth performance indicators of the feeding trial were individual weight, fork length and condition factor at the end of the 12-week feeding period. Average weight gain of treatment groups after the 12-week feeding trial was between 34.14 and 38.13 g. All diets (FM, PHP and SPH) were found to produce significantly heavier fish than the PL diet. Fish on the FM diet were also found to be significantly heavier than fish on the SPH diet (H (3) = 66.62, $p < 0.001$) but did not differ significantly to PHP fed fish (Table 6.3.). Fish in the group fed the FM diet were significantly longer than all other groups (H (3) = 122.2, $p < 0.001$, Table 6.3.). Condition factor was calculated using the fork length and total weight of each individual fish. Fish on the PHP diet had a condition factor of 1.32, which was significantly

greater than all other fish (H (3) = 158.8, $p < 0.001$, Table 6.3.). These results show that Atlantic salmon parr fed a low fishmeal/ high plant-protein diet grow significantly slower than fish fed a traditional diet higher in fishmeal. However, if the high plant-protein diet is supplemented with partly hydrolysed protein it can be used to grow Atlantic salmon parr as heavy, and of a better condition, than fish fed a traditional diet containing significantly more fishmeal.

3.2. Proximate composition

The whole-body proximate compositions of fish before and after the dietary treatment were analysed (Table 6.4.). Water content in fish sampled from all groups was not significantly different (F (4, 55) = 1.786, $p = 0.145$). Of the treated fish, the FM group had significantly higher ash content (2.14 ± 0.05%) compared to the fish on the other treatments (1.59 ± 0.04 – 1.74 ± 0.03%; F (4, 55) = 77.441, $p < 0.001$). The lipid content of fish on the SPH treatment was significantly reduced (9.55 ± 0.25%) compared to that of the pre-treatment (PT) fish (10.96 ± 0.27%; F (4, 55) = 4.495, $p < 0.01$). Finally, protein content in FM fish (17.26 ± 0.13%) was significantly greater than that of fish on the SPH treatment (16.76 ± 0.10%; F (4, 55) = 2.615, $p < 0.05$, Table 6.4.).

While total protein content only differed significantly between the two groups (FM and SPH), free amino acid concentrations in the blood samples showed multiple significant differences. Total free amino acids ranged from 3000.8 to 3533.6 μg mL^{-1} in the blood samples. SPH fish had the highest concentration of total free amino acids, while PL fish had significantly less (F (3, 40) = 5.636, $p < 0.01$). Significant variation in the level of free amino acids in the blood of fish on different dietary treatments was found for eight indispensable (arginine, histidine, lysine, methionine, threonine, valine, isoleucine, leucine) and eight dispensable amino acids (alanine, asparagine, GABA, glutamic acid, proline, serine, taurine, tyrosine; Figure 6.1.). For many amino acids, the SPH, followed by the PHP fish groups had the highest blood concentration levels (Figure 6.1.). However, in many of the indispensable amino acids PHP fish had the highest concentrations. PHP fish had significantly higher blood levels of the branched chain amino acids; valine, leucine and iso-leucine, compared to PL and FM fish and significantly higher levels of iso-leucine compared to SPH fish.

Table 6.3. The feed utilisation and growth performance of Atlantic salmon parr fed four different experimental diets for 12 weeks (FM, PL, PHP and SPH diets). Values with different superscripts in the same row are significantly different († = one-way ANOVA; $p < 0.05$, n=3, ± SD; * = Kruskal-Wallis H test and Duncan's Multiple Comparison test; $p < 0.05$, ± SD).

	FM	**PL**	**PHP**	**SPH**
Feed intake FI; g/fish†	**26.55** ± 1.17	**25.44** ± 1.88	**24.81** ± 1.03	**27.43** ± 0.50
Specific growth rate; %/day†	**1.61** ± 0.11	**1.44** ± 0.08	**1.57** ± 0.09	**1.52** ± 0.09
Feed conversion ratio†	**0.92** ± 0.09	**1.04** ± 0.06	**0.88** ± 0.07	**1.05** ± 0.09
Protein efficiency ratio†	**2.48** ± 0.26	**2.21** ± 0.13	**2.57** ± 0.13	**2.19** ± 0.20
Liver weight; mg†	**499.67** ± 17.73^{ab}	**425.29** ± 25.57^{a}	**561.67** ± 28.11^{b}	**484.21** ± 30.29^{ab}
Hepatosomatic Index; %†	**1.31** ± 0.03^{ab}	**1.25** ± 0.12^{a}	**1.52** ± 0.08^{b}	**1.35** ± 0.05^{ab}
Final weight; g*	**38.13** ± 2.84^{a}	**34.14** ± 3.23^{b}	**37.03** ± 0.88^{ac}	**35.71** ± 1.77^{c}
Final length; cm*	**14.39** ± 0.34^{a}	**13.87** ± 0.37^{b}	**13.92** ± 0.03^{b}	**14.00** ± 0.35^{b}
Final condition factor; k*	**1.23** ± 0.01^{a}	**1.24** ± 0.04^{a}	**1.32** ± 0.03^{b}	**1.26** ± 0.04^{c}

Table 6.4. The proximate compositions of salmon parr before dietary treatment (PT) and after 12 week feeding trial with four different experimental diets (FM, PL, PHP and SPH diets). Values with different superscripts in the same row are significantly different (one-way ANOVA; $p < 0.05$, n=3, ± SD).

	PT	**FM**	**PL**	**PHP**	**SPH**
Moisture	**72.16** ± 3.26	**70.43** ± 3.15	**72.81** ± 3.67	**72.12** ± 3.17	**74.24** ± 3.76
Protein	**17.04 ±** 0.47 ^{ab}	**17.26** ± 0.43 ^{a}	**16.82** ± 0.23 ^{ab}	**17.07** ± 0.57 ^{ab}	**16.76** ± 0.32 ^{b}
Lipid	**10.96** ± 0.89 ^{a}	**10.31** ± 0.67 ^{ab}	**10.14** ± 0.78 ^{ab}	**10.07** ± 0.79 ^{ab}	**9.55** ± 0.81 ^{b}
Ash	**2.46** ± 0.18 ^{a}	**2.14** ± 0.18 ^{b}	**1.74** ± 0.11 ^{c}	**1.58** ± 0.12 ^{c}	**1.63** ± 0.10 ^{c}

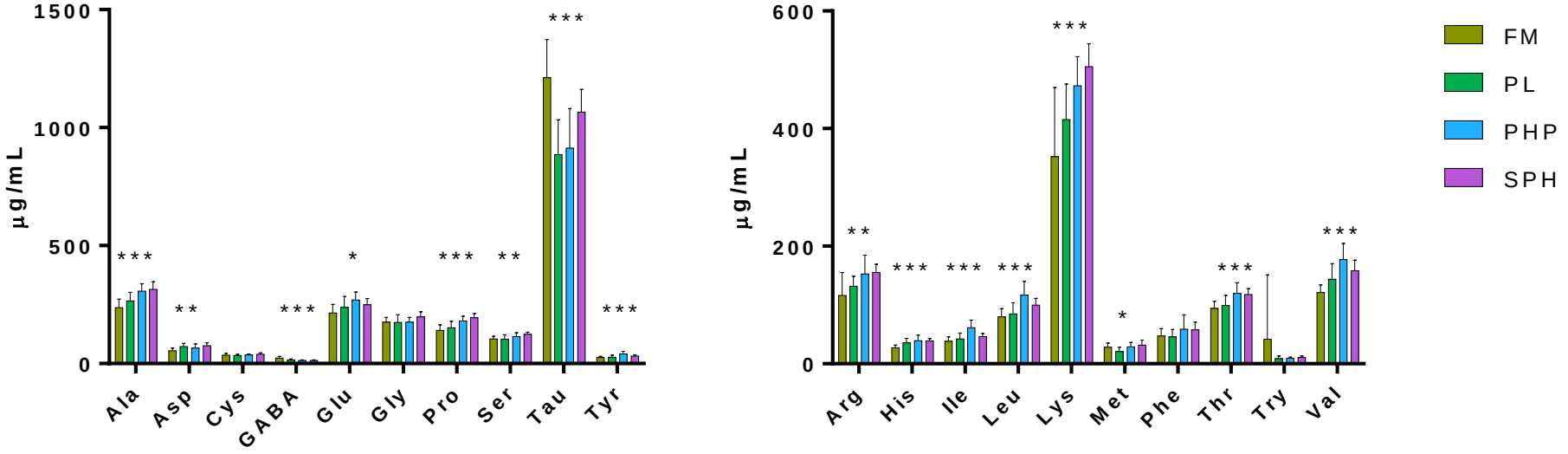

Figure 6.1. Blood amino acid concentrations. Dispensable (a.) and indispensable (b.) blood free amino acid levels in fish fed one of four diets; FM, PL, PHP or SPH. Values are means ± SD (*n*= 3). Statistical analysis was completed using one-way ANOVA and Tukey's Multiple Comparison test. Significant differences denoted as: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

3.3. Intestinal morphology

None of the fish evaluated in the present study showed signs of inflammation or enteritis in the gut (Figure 6.2.). Gut morphometrics were compared between treatment groups. SPH fish were found to have significantly wider villi (91.67 ± 20.33 µm) and wider intestinal walls (84.93 ± 23.62 µm) compared to FM fish (76.31 ± 14.92 µm and 58.97 ± 20.18 µm), PL fish (76.44 ± 22.63 µm and 63.18 ± 29.97 µm) and PHP fish (75.09 ± 14.89 µm and 65.57 ± 23.16 µm; nested ANOVA, $F(8, 72) = 3.384$, $p < 0.01$ and $F(8, 72) = 2.224$, $p < 0.05$, respectively). No significant differences were found for the other measurements (villi height, Vh/Vw ratio, intestine diameter, interior lumen area, internal perimeter, external perimeter and perimeter ratio) between the different dietary groups ($p > 0.05$; Table 6.5.).

Table 6.5. Gut histological morphometrics of salmon parr fed four different experimental diets for 12 weeks (FM, PL, PHP and SPH diets).

Values with different superscripts in the same row are significantly different (*nested or † one-way ANOVA; $p < 0.05$, $n = 3$, ± SD).

	FM	PL	PHP	SPH
Villi height (µm) *	**473.67** ± 130.92	**448.30** ± 227.62	**435.12** ± 159.02	**514.30** ± 161.04
Villi width (µm) †	**76.31** ± 14.92 [a]	**76.44** ± 22.63 [a]	**75.09** ± 14.89 [a]	**91.67** ± 20.33 [b]
Vh/Vw ratio †	**6.37** ± 1.97	**6.21** ± 3.35	**5.83** ± 1.80	**5.68** ± 1.60
Intestinal wall width (µm) †	**58.97** ± 20.18 [a]	**63.18** ± 29.97 [a]	**65.57** ± 23.16 [a]	**84.93** ± 23.62 [b]
Intestine diameter (mm) *	**2.23** ± 0.56	**2.10** ± 0.84	**2.08** ± 0.50	**2.43** ± 0.54
Interior lumen area (mm^2) *	**1.90** ± 1.18	**1.63** ± 1.30	**1.46** ± 0.68	**1.68** ± 0.71
Internal perimeter (mm) *	**24.73** ± 10.35	**21.13** ± 9.46	**25.16** ± 9.29	**27.15** ± 7.42
External perimeter (mm) *	**7.35** ± 1.88	**6.59** ± 2.47	**6.51** ± 1.50	**7.48** ± 1.50
Perimeter ratio (abs) *	**3.34** ± 0.81	**3.24** ± 0.88	**3.77** ± 0.62	**3.62** ± 0.55

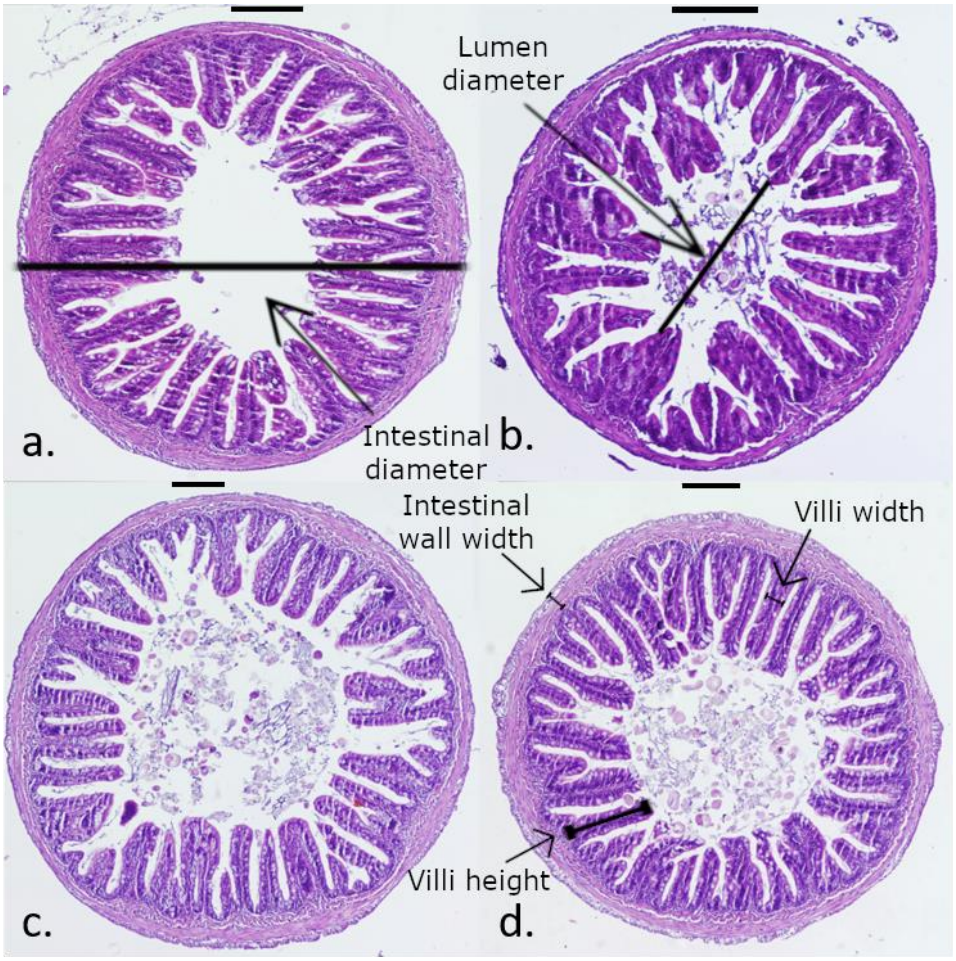

Figure 6.2. Example images of gut histology from fish fed four different treatment diets; FM (a.), PL (b.), PHP (c.), and SPH (d.). Blackline above each image represents scalebar = 200µm.

3.4. Gut microbiota composition

The bacterial composition of the intestinal contents of salmon parr, pre- and post- 12 weeks of dietary intervention, were analysed using 16S rRNA metagenomic sequencing. After quality filtering, there were a total of 7,910,986 reads, with reads per sample ranging from 31,266 – 205,661. Joining efficiency was 83.66 ± 0.14% and, after sequence clustering, 738 OTUs were identified.

Significant differences in α-diversity were found particularly between FM and the other treatment groups (Figure 6.3a. & b.). Chao1 diversity and Shannon indices were used to calculate species richness in the gut communities. In both indices, fish on the FM diet had a significantly higher diversity compared to all other groups ($p < 0.001$). Using the Chao1 diversity index, PT fish had a significantly lower diversity compared to fish on the FM diet ($F(4, 86) = 5.927$, $p < 0.05$, Figure 6.3a.). However, this difference became insignificant using the Shannon index (Figure 6.3b.).

β-diversity was visualised using a PCoA Bray Curtis plot and differences were calculated using permutational multivariate analysis of variance (PERMANOVA, 'Adonis') analysis (Figure 6.4.). Significant separation of the gut microbial compositions by treatment was found (PERMANOVA, $R^2 = 0.306$, $p < 0.001$). The samples from PT fish separated furthest from the other groups and clustered closely. Samples from fish fed the FM diet also clustered closely to each other, whereas the other three groups showed greater dispersion.

The microbial community compositions of the intestinal contents of the salmon parr were assigned to 14 phyla. However, > 90% of OTUs belonged to just five of these phyla. The relative abundance of the phyla identified from fish before the start of the dietary treatments was notably different from that of the fish at the end of the 12-week dietary intervention (Figure 6.5a.). PT fish were dominated by the phylum Deinococcus-Thermus (55%), followed by Firmicutes (19%) and Proteobacteria (12%). After the dietary treatments all fish had significantly higher relative abundance of Firmicutes ($F = 3.5$, $p < 0.05$) and Cyanobacteria ($F = 8.3$, $p < 0.001$) and lower Deinococcus-Thermus ($F = 31$, $p < 0.001$). The high abundance of Cyanobacteria observed possibly originated from the surrounding water or food and has been reported at similar levels in other studies investigating the gut microbiota of freshwater fish (Ye *et al.* 2014). The relative abundance of Proteobacteria did not change significantly, although it was highest in PHP followed by FM fish (Figure 6.5a.). The community composition

of FM and PT fish differed most significantly. FM fish also had a significantly higher ratio of Spirochaetes compared to fish fed the other three diets high in plant protein. Although all treatment groups were unique, the community composition was most similar between FM and SPH fish and, PL and PHP fish.

Analysis of the 16S data at family level showed that there was a core microbiota (OTUs present in ≥ 85% of samples; Llewellyn *et al.* 2016) shared across all treatment groups which involved *Streptococcaceae, Lactobacillaceae* and *Comamonadaceae* (Figure 6.5b.). *Thermaceae* were also in the core microbiota of PT, PL and PHP fish, and were found in 80% and 78% of FM and SPH fish, respectively. While *Bacillaceae* were in the core microbiota of PT, FM and PHP fish and were found in 80% of PL and SPH fish. PT fish had the most diverse core microbiota, which also included *Paenibacillaceae, Xanthomonadaceae, Blastocatellaceae* (Subgroup 4) and SHBZ1548 uncultured bacterium. FM fish also had the additional bacterial families; *Vibrionaceae* and *Peptostreptococcaceae* in their core microbiota. Fish fed the FM diet had a significantly higher ratio of the families *Moritellaceae, Psychromonadaceae, Helicobacteraceae* and *Bacteroidaceae* compared to fish fed the other three diets high in plant protein. At genus level PT fish were dominated by only six genera; *Meiothermus*, *Thermus*, *Anoxybacillus*, *Paenibacillus*, *Schlegelella* and *Pyrinomonas* (Figure 6.5c.). These genera remained part of the core microbiota for fish on the PL and PHP diet. The gut microbiota of fish post-dietary treatment were all dominated by the genus *Streptococcus* and fish fed diets high in plant protein also contained high relative abundances of *Aeromonas* (Figure 6.5c.). Comparing relative abundance of lactic acid bacteria, fish in the PT group had significantly lower levels compared to the dietary treated fish, while FM fish had significantly more compared to fish on diets high in plant protein (ANOVA, $p = 0.001$).

Fifty-seven OTUs were common to all groups post dietary treatment (Figure 6.6.). Of these, 33 were also found in PT fish. Nearly half of the shared OTUs were from the phylum Firmicutes, of which *Lactobacillus* and *Streptococcus* were the most commonly identified genera and were also found at high relative abundances.

Comparing the genera present in the dietary groups, PL fish had only one genus (*Devosia*) that was at a significantly different (higher) relative abundance compared to all other fish. PHP and SPH fish contained five and three genera, respectively, at a significantly different relative abundance compared to all other fish. FM fish had a significantly higher

relative abundance of 16 bacterial genera compared to the other three diets high in plant protein.

In the shared microbiota, found in all fish, there were several genera and species identified which, to the best of our knowledge, from reviewing published literature, have not been previously found in fish. These include *Enterococcus timonensis*, *Lactobacillus salivarius* and *Terrisporobacter* sp. These OTUs were not detected in the sequenced negative control.

Analysis of the 16S microbial composition of the intestinal contents of salmon parr before and after a 12-week dietary treatment has highlighted numerous differences. Overall, younger PT fish harbour a diverse community that is dominated by the phylum Deinococcus-Thermus and show low inter-sample variation. After the 12-week dietary treatment Firmicutes became the dominant phylum. FM fish had the highest alpha diversity, while greater variation in inter-sample diversity and community composition was seen in the fish under the other dietary treatments that had significantly higher plant-protein content.

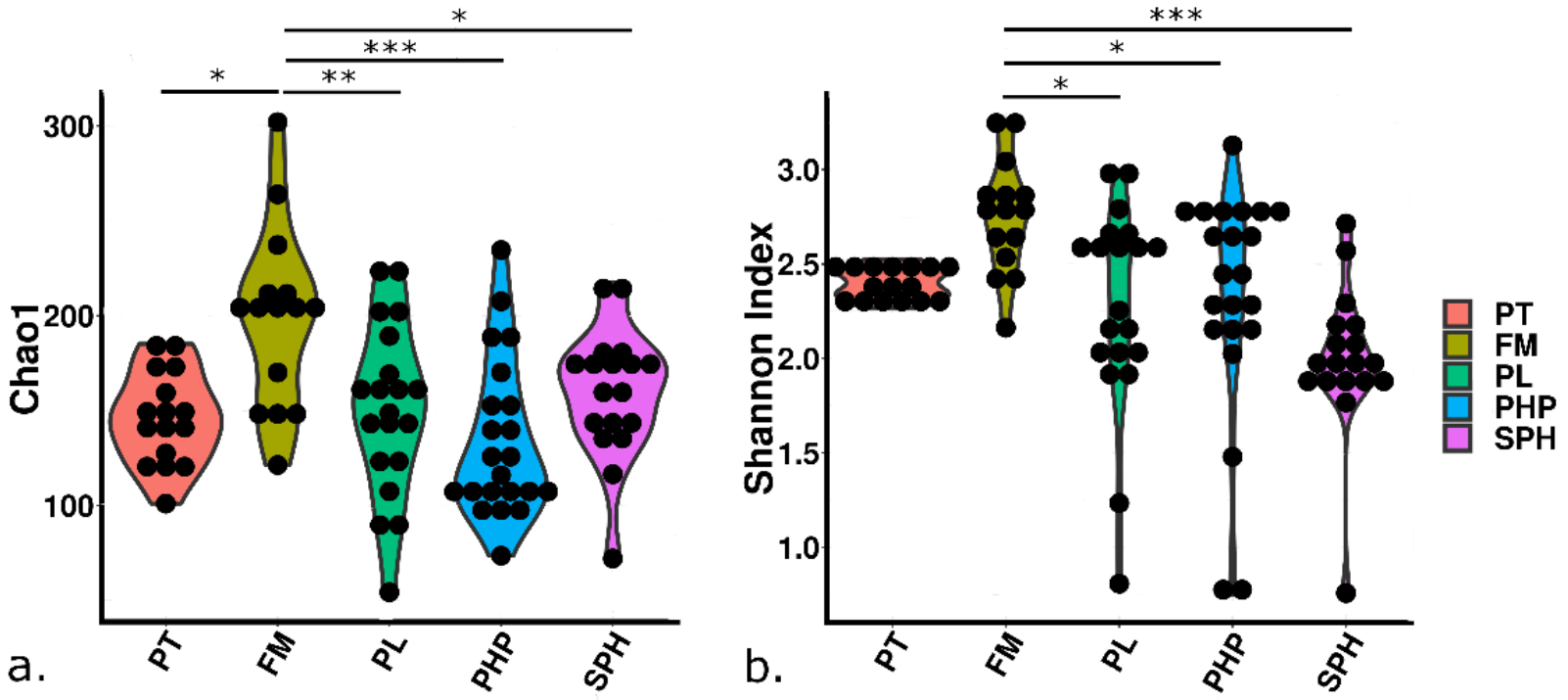

Figure 6.3. α-Diversity measures; Chao1 index (a.) and Shannon index (b.), of 16S microbiota gut community compositions of pre-treatment salmon parr (PT) and fish that were fed four different dietary treatments (FM, PL, PHP and SPH) for 12 weeks. Significant differences denoted as: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$),

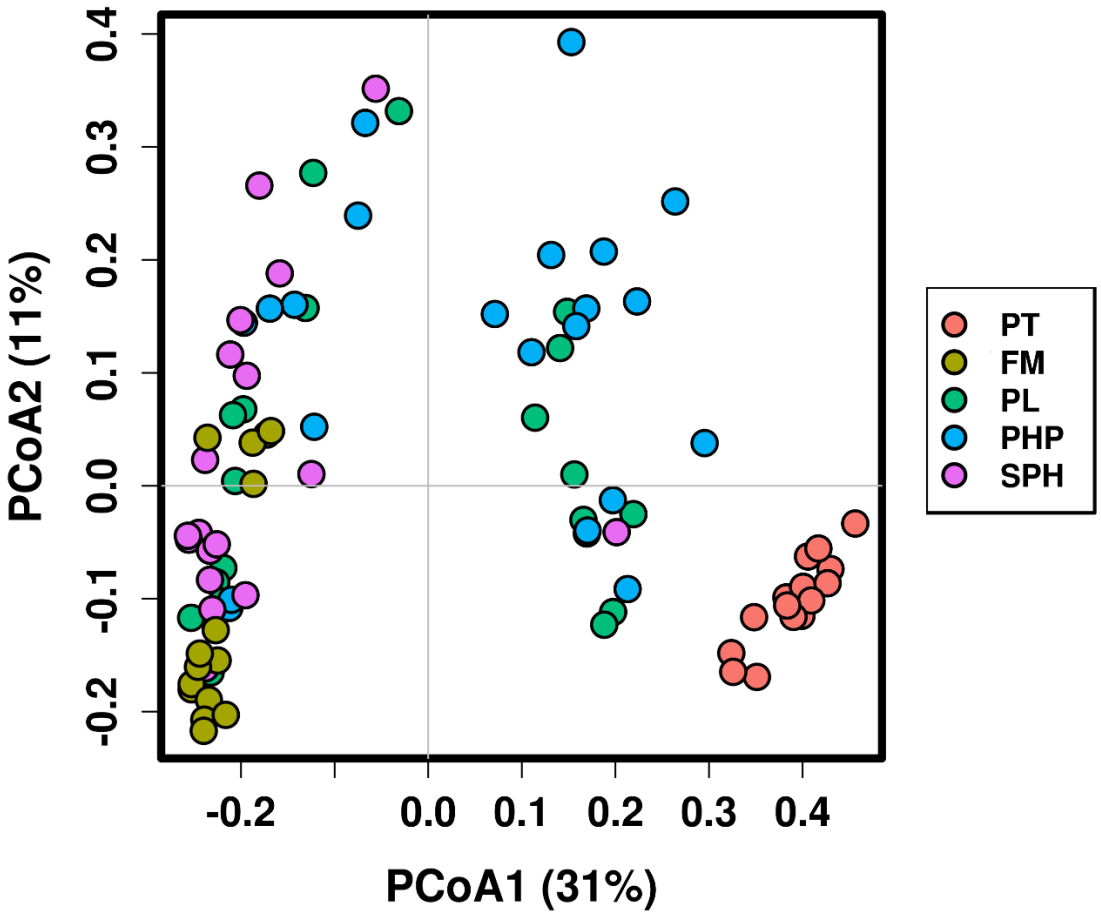

Figure 6.4. PCoA Bray Curtis plot of 16S OTU relative abundances associated with pre-treatment salmon fish (PT) and fish that were fed four different dietary treatments (FM, PL, PHP and SPH) for 12 weeks (*n*=3). The first and second principal component explained 42% of the sample variations.

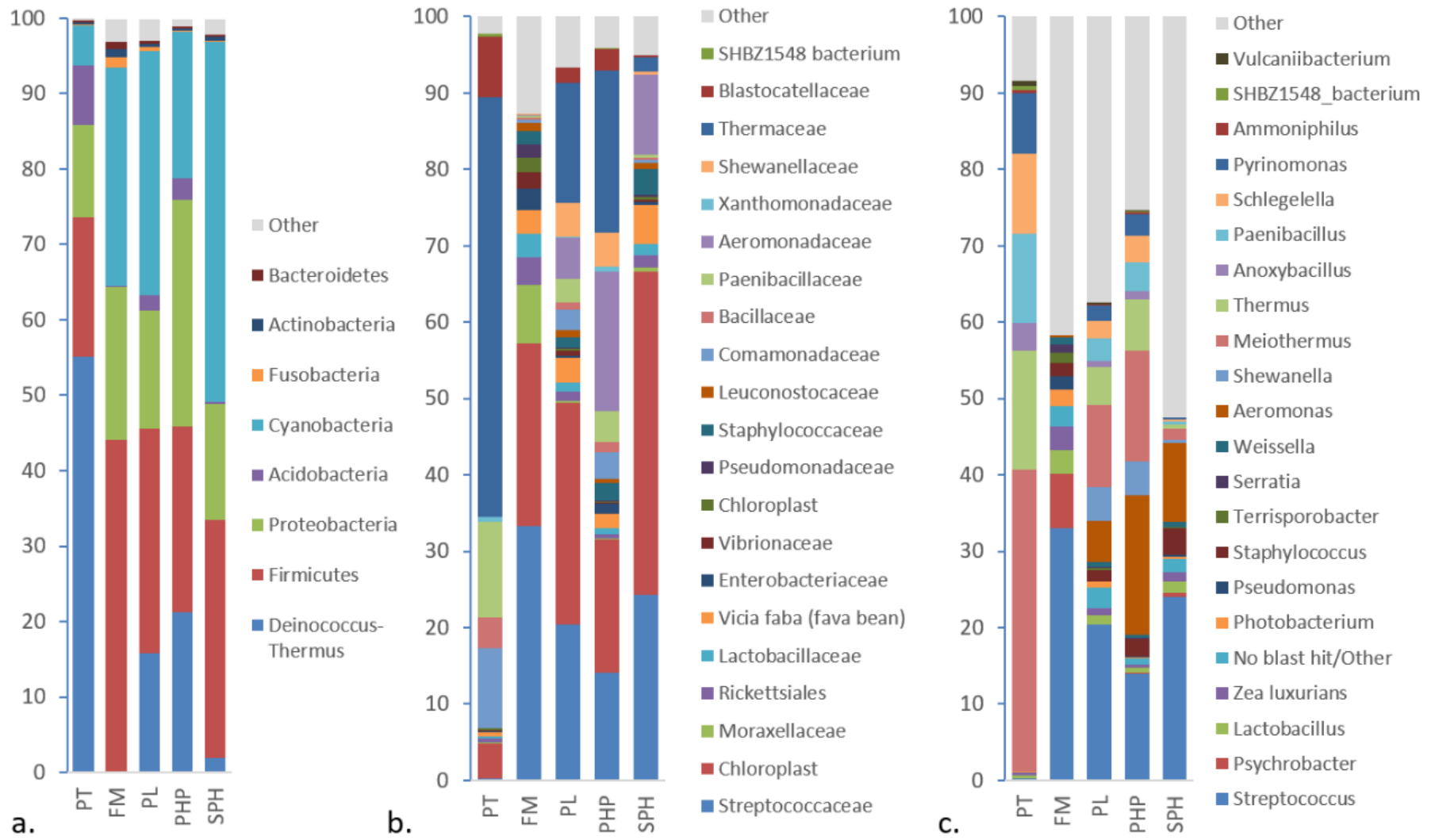

Figure 6.5. 16S OTU relative abundance at the phylum (a.), family (b.) and genus (c.) level for pre-treatment salmon parr (PT) and fish that were fed four different dietary treatments (FM, PL, PHP and SPH) for 12 weeks.

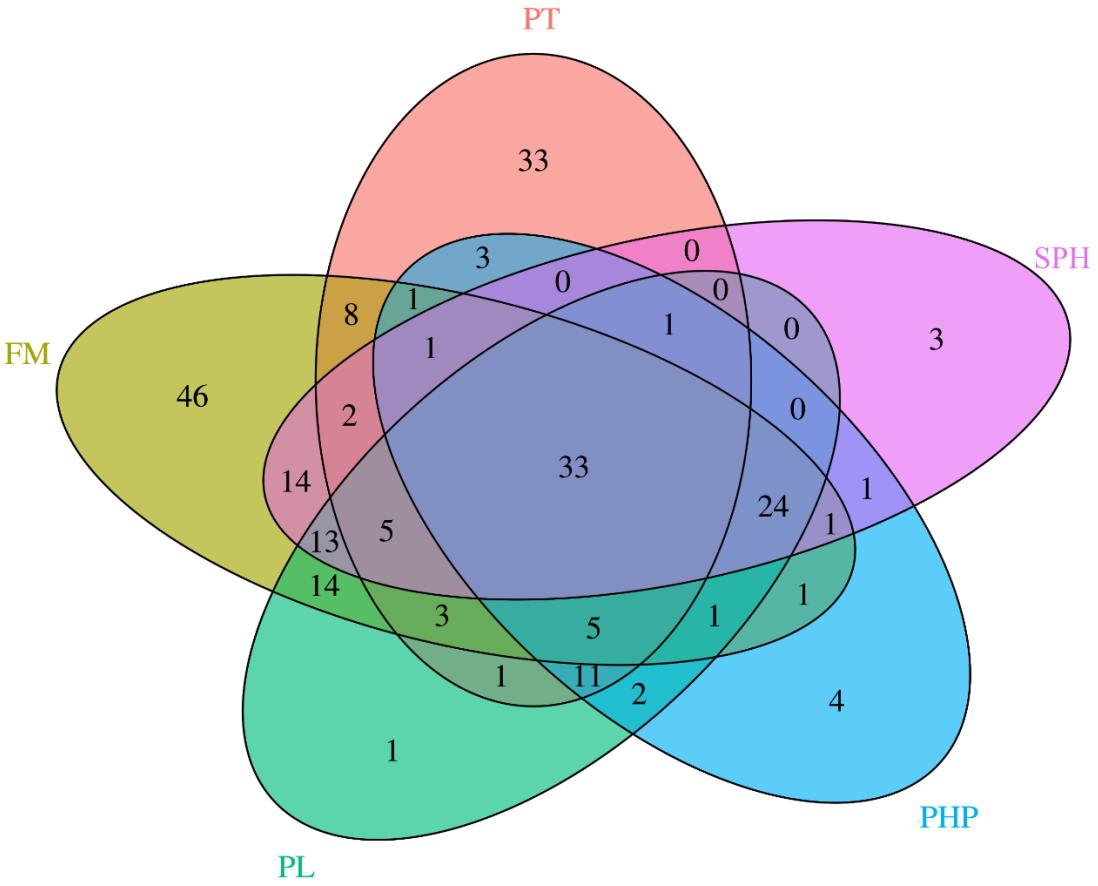

Figure 6.6. Total number of shared OTUs present in the intestinal contents of all fish in the pre-treatment (PT) and 4 dietary treatment (FM, PL, PHP and SPH) groups (*n*=3).

3.5. Cost comparison

Often the cost of supplements required to sufficiently replace the nutrients derived from marine-origin resources are costly and considered un-economically viable. A cost comparison was carried out as part of this study to compare the variable protein ingredients in each of the four diets. The cheapest feed was the PL diet at €432.90 metric tonnes $(MT)^{-1}$. The protein in the FM and PHP diets cost a similar amount; €540.58 MT^{-1} and €541.56 MT^{-1}, respectively, while the SPH feed was the most expensive at €675.19 MT^{-1} (Table 6.1.).

4. DISCUSSION

This study has shown that reducing the fishmeal component of feeds, from 35% to 15%, in the place of plant proteins (PL diet), resulted in reduced growth in Atlantic salmon parr. Whereas, reducing significantly the fishmeal component from 35% to 5% while supplementing the diet with 10% partly hydrolysed FPH (PHP diet) allowed similar growth performance compared to fish on the traditional fishmeal diet (FM). The four experimental diets were prepared to match at all levels of nutrition, except for protein source. Their amino acid profiles were similar, and all provided essential amino acids at levels above requirements (Table 6.2.). Despite this, after 12 weeks of feeding Atlantic salmon parr four different diets, significant differences in final weight and fork length were recorded at an individual level. Specific growth rate, feed conversion ratio and protein efficiency ratio results follow the trend of highest growth rates in FM and PHP fish. Interestingly, the hepatosomatic index in PHP fish was significantly higher than PL fish. During salmon production, it is ideal to have parr at maximum size and optimum health in advance of smoltification. During smoltification, a period of high energy requirements, whole-body and liver lipid reservoirs become depleted (Nordgarden *et al.* 2002). Atlantic salmon parr with higher levels of stored lipids may have increased energy for smoltification, which may, in turn, prevent a 'protein sparing effect' and, ergo, result in healthier, larger smolts.

While aquaculture production has expanded exponentially, fishmeal production has remained stagnant. Plant-derived proteins have largely been used to make up the increased demand for protein in aquafeed production. Soybean meal, a by-product of soybean oil production, has been the most popular source; regarded as economical and relatively nutritious, with high crude protein content and a sufficiently suitable amino acid profile (Gatlin *et al.* 2007). Soybean meal has been successfully used to replace fishmeal in aquafeeds for omnivorous fish (El-Saidy and Gaber 2002; Lin and Luo 2011; Kumar *et al.* 2017). However, replacement of fishmeal with soybean meal in diets for carnivorous finfish has been more challenging due to problems with digestion, utilisation, anti-nutritional factors and possible absence of essential but unknown species-specific growth factors (Blaufuss and Trushenski 2012; Turchini *et al.* 2019). Anti-nutritional factors include compounds such as protease inhibitors, phytate, saponins, lectins, oligosaccharides, and various allergenic compounds that negatively affect feed consumption, digestibility, absorption and bioavailability of nutrients

and can cause intestinal damage and alterations to gut microbial communities (Francis *et al.* 2001; Merrifield *et al.* 2011; Blaufuss and Trushenski 2012). Soybean meal protein concentrate can be made from soybean meal by aqueous alcohol extraction from defatted soybeans. This process reduces anti-nutritional factors significantly. As a result, soybean meal protein concentrate has been successfully used to replace up to 50% fishmeal protein in carnivorous fish diets, while maintaining rapid growth rates and feed efficiency values (Hardy 2010; Davis 2015). However, beyond this inclusion level, problems with feed intake and growth are reported (Blaufuss and Trushenski 2012).

In this study, the fish fed the PL diet did not show a difference in feed intake but grew significantly less than the other dietary groups (4.6 – 11.7% smaller fish at the end of dietary intervention). The blood concentration of total free amino acids in these fish was the lowest of all the groups (5.8 - 17.8% less). These results suggest that anti-nutritional factors related to the high level of soybean meal protein concentrate in the PL diet resulted in reduced blood amino acid concentrations and growth performance. It was noted, however, that although many of the blood free amino acids in the PL fish were significantly lower than those recorded in PHP and SPH fish, this was not the case for FM fish. Hence, plant-related anti-nutritional factors may have also inhibited the absorption of other nutrients, such as macro and trace metals, and lipids, abetting growth inhibition, as seen previously (Gatlin III and Phillips 1989; Yousif *et al.* 1994; Usmani and Jafri 2002).

Protein requirements are related to amino acid bioavailability and, it has been shown here and in numerous previous studies that plant-derived protein is of inferior quality in terms of digestibility and bioavailability compared to fishmeal (Mambrini *et al.* 1999; Kaushik *et al.* 2004; Parisi *et al.* 2004; Espe *et al.* 2006; Collins *et al.* 2013). However, our results have shown that supplementation of a predominantly plant protein diet containing only 5% fishmeal with partly-hydrolysed FPH (PHP supplement) is as effective as a 35% fishmeal diet. Indeed, despite consuming similar levels of feed, fish on the PHP diet were significantly heavier than PL fish and had a better condition factor compared to PL fish and as well as FM fish (in terms of final weight for the latter). FPH are considered excellent ingredients for aquafeeds due to their nutritional value and functional and bioactive properties (Khosravi *et al.* 2015). Their nutritional and health-promoting characteristics are due to their significant fractions of single amino acids and low-molecular-weight peptides that are generally easier to digest and absorb. Interestingly, it is now known that many amino acids are more rapidly and efficiently

absorbed as di- and tripeptides, rather than single amino acids (Conceicao *et al.* 2012). In this study, although SPH fish had, overall, the highest concentration of total free amino acids in their blood, PHP fish had the highest levels of essential amino acids. The blood levels of branched chain amino acids of PHP fish were at a significantly higher concentration than FM and PL fish and this remained true for SPH fish in terms of isoleucine. Branched chain amino acids play important structural roles and act as an anabolic signal for protein synthesis (NRC 2011). The results of this study suggest, increased bioavailability of essential amino acids, especially branched chain amino acids, as indicated by blood amino acids concentrations, stimulated increased protein synthesis and growth in the fish. This finding has been previously reported in Atlantic salmon at different life stages and in other aquaculture species also (Berge and Storebakken 1996; Fournier *et al.* 2004; Refstie *et al.* 2004; Hevrøy *et al.* 2005; Espe *et al.* 2006; Espe *et al.* 2007; Khosravi *et al.* 2015). However, studies have found that results are dose-dependent and negative effects can occur from higher levels of inclusion (Refstie *et al.* 2004; Hevrøy *et al.* 2005; Espe *et al.* 2012). Although PHP and SPH were supplemented at the same concentration of total feed, the lower molecular weight proteins that make up the SPH may have meant it acted like a higher dose of hydrolysate, and therefore, similar to previous studies, resulted in reduced effectiveness for growth stimulation. The mechanistic reasons behind these effects warrant further investigation.

FM fish did not show as high levels of blood amino acids as PHP fish (3174.2 ± 286.8 vs. 3357.4 ± 298.1 μg mL^{-1}), however, their growth performance (FCR, SGR, PER, HSI and final weight) was on par. It has been reported previously that fishmeal contains unknown growth factors, which have been since identified as naturally occurring trace and ultra-trace compounds such as amines and steroids (Hardy 2010; Turchini *et al.* 2019). This suggests that alternative pathways, beyond protein absorption, were stimulating high growth rates in these fish.

Gut morphology and gut microbial community composition were investigated. Inflammatory or degenerative changes in the gut, indicative of soy-induced enteritis, were not present in any histological section from the fish examined. The only significant differences recorded were wider villi and thicker intestinal walls in SPH fish. Larger villi (higher and wider) equate to more and/or bigger cells, providing greater surface area for absorption of nutrients and metabolites. This is supported by SPH fish having the highest level of total blood amino acids. It could be hypothesised that this morphological characteristic would promote growth.

However, this was not seen here and similarly reported not to correlate elsewhere (de Verdal *et al.* 2010).

16S rRNA sequencing revealed that fish from each dietary group had distinct gut microbial communities. β-diversity analysis of the microbiota in the intestinal contents of the fish revealed a clear and significant separation of FM fish from fish on the other three diets high in plant protein. Dietary protein source has previously been reported to alter gut microbiota composition (Hartviksen *et al.* 2014; Gajardo *et al.* 2017). Zarkasi *et al.* (2016) compared the microbiota of Atlantic salmon fed a commercial (35% fishmeal) diet and diets that were low in protein or low in fishmeal. Similar to this study, their β-diversity plots show the pre-treatment fish and the fish fed the commercial fishmeal diets clearly separating from the other experimental groups. This same trend was seen in a study by Gajardo *et al.* (2017).

Fish on the FM diet had significantly higher α-diversity. Fifty-four OTUs were found only in this group and they also had a significantly higher relative abundance of 16 shared OTUs, primarily from the Proteobacteria and Firmicutes phyla. In adult humans and mammals, a diverse microbiota has frequently been linked with a balanced, well-functioning metabolism (Ley *et al.* 2005; Turnbaugh and Gordon 2009; Menni *et al.* 2017). It has been shown in a number of studies that the gut microbiome affects metabolism in fish; affecting nutrient uptake (Semova *et al.* 2012), metabolism pathways (Ni *et al.* 2014) and ultimately growth (Ye *et al.* 2011). Furthermore, Webster *et al.* (2018), comparing the gut microbiota of Atlantic salmon parr sourced from hatcheries and from the wild, found, overall, wild populations had considerably higher microbial diversity than hatchery populations. The interaction of the gut microbiota with dietary components is complex and multifaceted. However, it is likely that the increased microbial diversity recorded in the intestinal contents of FM fish enhanced nutrient uptake and metabolism beyond that of protein absorption.

Comparing gut microbial composition of fish fed the diets high in plant protein to the fishmeal control diet, bacterial families known for their proteinaceous metabolic activity (e.g. *Vibrionaceae, Peptostreptococcaceae, Pseudomonadaceae, Moraxellaceae*) were reduced in the place of increased carbohydrate or broad range metabolisers (e.g. *Thermaceae, Shewanellceae, Aeromonadaceae*). Interestingly, these alterations were not as significant in fish supplemented with the SPH. Thus, data suggests that the gut bacterial community adapted to the altered diets.

There have only been a few studies investigating the gut microbial composition of Atlantic salmon at the freshwater stage (Navarrete *et al.* 2009; Llewellyn *et al.* 2016; Dehler *et al.* 2017; Rudi *et al.* 2018). From these studies core or principal phylotypes reported included Firmicutes, Proteobacteria, Actinobacteria and Tenericutes, as well as Clostridiales, *Mycoplasmataceae, Enterobacteriaceae, Comamonadaceae, Ruminococcaceae, Microbacteriaceae, Hyphomicrobacteriaceae, Peptostreptococcaceae, Yersinia, Vagococcus, Acinetobacter, Shewanella, Microbacterium, Cellulomonas, Serratia, Pseudomonas, Chryseobacterium., Staphylococcus, Escherichia/Shigella, Brucella* and *Corynebacterium* among others. In this study we also report significant levels of Firmicutes, Proteobacteria, *Enterobacteriaceae, Comamonadaceae, Peptostreptococcaceae* and *Shewanella.* Notably absent are significant levels of Tenericutes, *Mycoplasmataceae* and *Pseudomonas.* The small number of studies to date and the wide variation in results reported means that what constitutes a healthy microbiota for Atlantic salmon parr has yet to be fully defined. Without this knowledge it is difficult to accurately determine to effects of dietary treatment. While no obvious increase in pathogenic or harmful bacteria were noted a significant decrease in lactic acid bacteria were recorded in fish on high plant protein diets compared to the fishmeal control fish.

FPH can be produced from fish processing by-products, fishery by-catch, and low-value pelagic species not currently directly consumed by humans. When using sustainable processing practices, it can be one of multiple product streams derived from suing the entire fish. Its high nutritional value allows it to be used in small quantities as a supplement to fortify diets. These characteristics suggest that its use could go some way towards sustainable food production and reducing the volume of wild fish species used in aquafeeds, an important consideration in light of recent biodiversity reports (IPBES 2019). This study has shown that farmed Atlantic salmon parr can grow successfully on an 80% plant protein diet when supplemented with a partly-hydrolysed FPH (PHP). PHP fish also had relatively high heptosomatic indices, possibly indicating higher liver lipid stores that would benefit fish during smoltification. Furthermore, a cost comparison of the different feeds highlighted that this formulation is an economically viable alternative. The results indicate that improved essential amino acid bioavailability, in particular branched chain amino acids, facilitated the high growth rates recorded in PHP fish. Although the study reports some significant results, not all growth performance indicators revealed statistically significant differences. Further

research into the effects of the different diets over extended periods, and associated variations in gut microbiota using metabolomics and shotgun sequencing to ascribe digestive roles would be beneficial to gain a greater understanding of the interaction of dietary nutrients and gut microbiota and their effects on host health and development.

Acknowledgements

We would like to gratefully acknowledge the funding of this work by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. via the IRC Enterprise Partnership Scheme and Teagasc, Ireland (Project I.D. EPSPG/2015/57). The authors are supported in part by Science Foundation Ireland in the form of a centre grant (APC Microbiome Ireland Grant No. SFI/12/RC/2273) and by the Science Foundation Ireland Investigators Award 15/IA/3028; the Sea Change Strategy, NutraMara programme (Grant-Aid Agreement No. MFFRI/07/01); and the SMART FOOD project: 'Science Based 'Intelligent'/Functional and Medical Foods for Optimum Brain Health, Targeting Depression and Cognition' project (Ref No. 13/F/411) with the support of the Marine Institute and the Department of Agriculture, Food and the Marine (DAFM) in Ireland. We are grateful to Mr Peter McGovern, Salmon Springs Galway Ltd. for use of facilities and technical advice and to Dr Jónas Jónasson, Stofnfiskur, Iceland for the kind donation of the salmon eggs. We acknowledge the Teagasc Sequencing Facility, Dr Fiona Crispie, Dr Paul Cotter and Ms. Laura Finnegan for their technical assistance with the 16S rRNA MiSeq sequencing. Furthermore, we would like to acknowledge and thank Kieran Kelly, Victoria Molloy, Clodagh Carr, Charlotte Bolton, John Hyland, Erwin Vinke, Katinka Vandewalle, Dr Joshka Kaufmann and Dr Liz Ryder for their input into training, fish husbandry and sample collection.

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Chapter 7

Investigating the potential of a PUFA rich fish oil as a nutraceutical in an animal model of early life stress

Under review: *Nutritional Neuroscience* (Jan 2020)

ABSTRACT

Early life stress is a key predisposing factor for depression and anxiety disorders. There is a growing need for new nutraceutical-based strategies to blunt the effects of adverse-life events. To this end, the maternal separation model in rats was used to test the efficacy of fish oil dietary supplementation, on its own and in conjunction with the anti-depressant fluoxetine, as a treatment for depressive and anxiety-like symptoms associated with early life stress. Behavioural tests (open field test, plus maze test and forced swim test) and biochemical markers (corticosterone, BDNF, brain fatty acids and short chain fatty acids) were used to analyse the effects of the dietary treatments. Maternally separated rats showed depressive-like behaviours in the forced swim and open field tests. These behaviours were prevented significantly by fluoxetine administration, and in part by fish oil supplementation. Fish oil supplementation significantly increased docosahexaenoic acid (DHA) levels in the brain and altered the omega-6: omega-3 ratio. Other associated biochemical changes reported include altered brain fatty acids, significantly lower plasma corticosterone levels (AUC) and reduced brain stem serotonin turnover, compared to untreated, maternally separated (MS) rats. Gut microbial communities and relating metabolites (SCFA) were analysed to investigate possible changes in the gut-brain axis. Untreated MS animals had significantly lower ratios of SCFA producers such *as Caldicoprobacteraceae, Streptococcaceae, Rothia, Lachnospiraceae_NC2004_group*, and *Ruminococcus_2*, along with significantly reduced levels of total SCFA. Animals fed fish oil had significantly higher Bacteroidetes and *Prevotellaceae* and reduced levels of butyrate. While fluoxetine treatment resulted in significantly higher levels of *Neochlamydia, Lachnoclostridium, Acetitomaculum* and *Stenotrophomonas* and acetate and propionate.

1. INTRODUCTION

The brain is the most complex and metabolically demanding organ in the human body. Brain disorders are amongst the most devasting medical conditions. It is estimated that as many as 970 million people globally suffer from a mood or substance use disorder (Ritchie and Roser 2019). Mood disorders are diverse and can vary greatly in their symptoms and co-morbidities. The multi-faceted and complex nature of these disorders has meant that knowledge about their pathophysiology is rudimentary compared with other common chronic, multifactorial conditions (Krishnan and Nestler 2008). However, research in this area continues to work towards deciphering the underlying causes of the symptoms.

Selective serotonin re-uptake inhibitors (SSRIs), such as citalopram, paroxetine and fluoxetine, are pharmaceutical compounds used as the first line of treatment for depression. Although SSRIs frequently produce positive results, they do not work for all patients. In-fact studies have found that their success rate is approximately 30 – 50% (Trivedi *et al.* 2006; Vieta and Colom 2011). Furthermore, patients on SSRIs can experience negative side effects and adverse drug reactions (Kulkarni and Dhir 2009; Cryan and Leonard 2010). Therefore, there is significant interest in finding new antidepressant agents possessing better efficacy and minimal side effects, as well as identifying appropriate and effective natural dietary interventions and novel adjunct therapies.

Fish oil is high in the bioactive compounds omega-3 polyunsaturated fatty acids (PUFA), in particular docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Today, consumption of fish oil is associated with different health benefits, the principle ones being improved cardio-vascular health, as well as healthy brain and eye function. A culmination of results from animal and clinical trials illustrate the beneficial effects of omega-3 PUFA on cognitive function, especially in developing, aged or cognitively impaired cohorts (Luchtman and Song 2013). Nonetheless, the efficacy of EPA and DHA treatment for mental disorders has been questioned over the years, with reviews finding that positive results can be isolated to particular cohorts or only present a positive trend rather than significant results (Sontrop and Campbell 2006; Mello *et al.* 2014). However, new research is continually uncovering the multi-faceted mechanisms by which omega-3 PUFA influence neuro-chemical pathways and alter behaviour (Song *et al.* 2008; Vines *et al.* 2012; Gilbert *et al.* 2014). Research from our group has shown that patients suffering from major depression have higher omega-6: omega-

3 fatty acid status in red blood cell membranes and plasma, coupled with elevations in pro-inflammatory cytokines, compared with healthy controls (Dinan *et al.* 2009). Using *in-vitro* techniques researchers from our lab showed how DHA reverses glucocorticoid hormone corticosterone-induced neuronal apoptosis and attenuated glucocorticoid hormone corticosterone-induced reductions in brain derived neurotrophic factor (BDNF) mRNA expression (Pusceddu *et al.* 2015). Moreover, in an animal model our research group found that neuro-behavioural development related to cognitive, anxiety and social behaviours, is highly dependent upon *in utero* and lifelong omega-3 PUFA availability (Robertson *et al.* 2017). The bioactive characteristics of these dietary nutrients are influential, at least in part, via the gut-brain axis.

This axis, now adjusted to the microbiota-gut-brain axis, has received significant attention in recent years (Dinan and Cryan 2017a; Mayer and Ryu 2019). Research has uncovered how it provides multiple mechanistic pathways by which dietary compounds and microbial metabolites affect neurobiology and how in turn alterations in neurochemistry influence microbial composition and the workings of the gut (Zhou and Foster 2015; Kelly *et al.* 2017; Sherwin, Dinan *et al.* 2018). The pathways of communication include the enteric and autonomic nervous system and many different classes of molecules such as microbial metabolites like short chain fatty acids (SCFA), neurotransmitters (e.g. GABA and dopamine) and inflammatory cytokines (Leclercq *et al.* 2019). Understanding the specifics and dependencies of the cross talk between the axis components will facilitate the development of dietary strategies and novel therapies to improve healthy brain development and functions.

SCFAs (primarily acetic, propionic and butyric acid) are important energy sources for gut microbiota and host intestinal epithelial cells, but also modulate physiological functions, including absorption, motility, mucus release and gut epithelial cell proliferation (Sakata 2019). Furthermore, they can regulate immunity in multiple ways such as increasing the number of regulatory T cells, inhibiting nuclear factor-κB (NF-κB), and activating production of anti-inflammatory cytokines (Rooks and Garrett 2016).

Adverse events at a young age or early life stress (ELS) are known to contribute to a predisposition to the development of disease, including multiple psychiatric disorders, later in life. During this critical developmental period (e.g. perinatal life) neurobiological systems implicated in regulating emotion and stress responses are being programmed and the level

of responsiveness is established for later life. ELS can dysregulate this development, often leaving the individual maladapted to handling stress and prone to depression. Studies have linked ELS to a wide spectrum of physiologic alterations including epigenetic alterations, inflammatory changes and dysregulation of the hypothalamic pituitary adrenal axis (HPA; Syed and Nemeroff 2017). The maternal separation model in rats has been shown to produce animals with behavioural, biological and microbial abnormalities typical of depressed people who have previously experienced ELS (O'Mahony *et al.* 2011).

This study thus aimed to investigate nutritional approaches for avoiding or treating depression by comparing the impact of dietary fish oil supplementation on its own or as an adjunct therapy, with the administration of the SSRI anti-depressant fluoxetine, in the maternal separation model of early life stress. We used behavioural tests and biological markers of depressive and anxiety-like symptoms to assess the effects of maternal separation with and without dietary treatment (administered fish oil, fluoxetine or a combination of fish oil and fluoxetine) on rats. We then compared these results with 16S microbial composition and caecal SCFA levels to investigate possible mechanistic pathways through which the different treatments impacted.

2. MATERIALS AND METHODS

2.1. Diets & treatment groups

There were five treatment groups; a non-maternally separated control group (NS-Con) that were fed a standard chow diet (E15712-04; *n* = 12), a maternally separated control group (MS-Con) fed the standard chow diet (*n* = 12), a maternally separated group fed a 0.015% fluoxetine (PHR1394, Sigma Aldrich Ireland Ltd., Wicklow, Ireland) supplemented diet (MS-FX; *n* =12), a maternally separated group fed a fish oil supplemented diet (MS-FO; *n* = 10) and a maternally separated group fed a diet supplemented with fluoxetine and fish oil (MS-FX-FO; *n* = 12). All diets, formulated by Ssniff Spezialdiäten GmbH (Soest, Germany), were isoenergetic and matched for macronutrient content, differing only in fatty acid profile and presence or absence of fluoxetine. The detailed macronutrient and fatty acid compositions of the diets are shown in Tables S7.1. and S7.2. The concentration for fluoxetine was calculated based on doses previously reported in animal model studies and considering the average daily food intake and body weight of Sprague Dawley rats aged between 9 and 16 weeks (Laaksonen *et al.* 2013). Estimated dose for fluoxetine was 10 mg/kg/day (Arndt *et al.* 2015). Fish oil was added to the diets in the place of soybean oil in the standard chow, at 7% of total feed. Calculation for DHA dose was calculated considering the average daily food intake and body weight of Sprague Dawley rats aged between 9 and 16 weeks (Laaksonen *et al.* 2013). Estimated dose of DHA was 467 mg/kg/day.

2.2. Experimental design

The experimental design is illustrated in Figure 7.1. Male and female Sprague-Dawley rats were obtained from Envigo, UK and mated in the local animal unit (UCC, Ireland). Food and water were available *ad libitum* and animals were maintained on a 12:12-h dark—light cycle with temperature at 21 ± 1 ^{o}C. Pups of the resulting litters were randomly assigned to five treatment groups. Four groups underwent maternal separation as previously described (O'Mahony *et al.* 2008; Pusceddu *et al.* 2015). This involved separating the rat pups from their dams as a whole litter for a period of 180 min by placing them in a plastic cage on top of heater pads (30—33 ^{o}C) in a room separate to the main holding room. Maternal separation took place daily from postnatal day (PND) two to 12 between 0900 h and 1200 h. The control group consisted of non-handled pups, left untouched with their respective dams. After

postnatal day 12, rats were left undisturbed except for routine husbandry. From PND 22, male offspring were weaned onto the same diets as their mothers. Subsequently, they were group-housed (2 – 4) in large cages with sawdust bedding and an enriched environment. From week eight the treatment groups were fed their assigned treatment diet. After two weeks on the treatment diets, the male offspring underwent a battery of behavioural tests over a period of 4 weeks. Behavioural tests were conducted in sequence from the least to the most stressful test. At 16 weeks, animals were sacrificed by decapitation and tissue samples were rapidly harvested. All experiments were approved by the Ethics Committee of University College Cork, licenced by HPRA, Ireland and in full accordance with the European Community Council Directive (2010/EU/63).

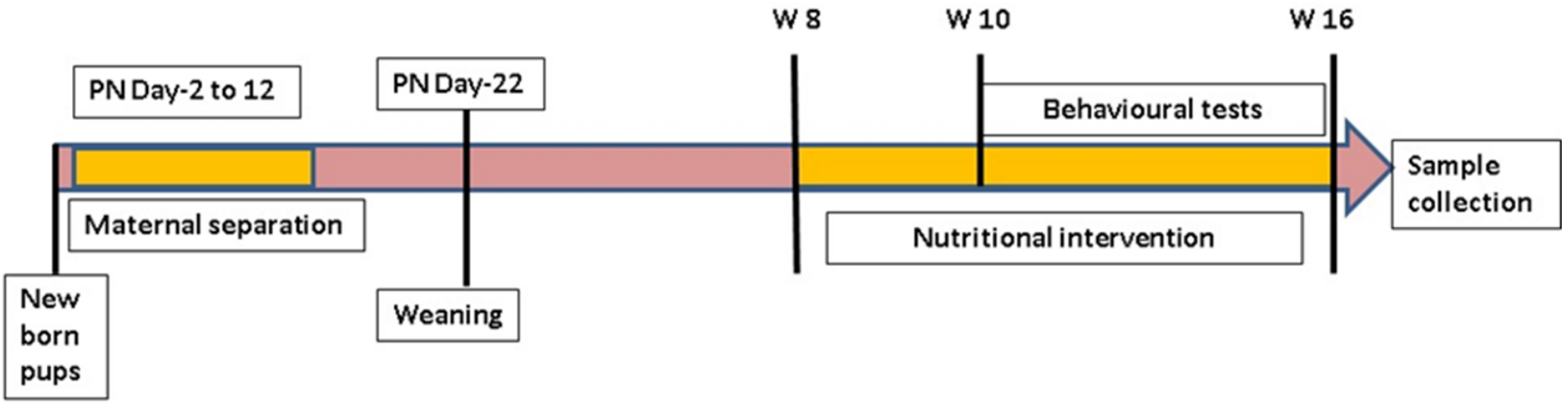

Figure 7.1. Experimental design outlining key stages. Maternal separation took place between postnatal day (PND) two to 12. Weaning began at PND 22. At week eight dietary intervention began and continued until culls at week 16. Behavioural tests were carried out between week 10 and week 16.

2.4. Behavioural testing

2.4.1. Elevated plus maze

The elevated plus maze is a common test to assess anxiety in rodents. It was performed as previously described (Cryan *et al.*, 2004). Briefly, the maze consisted of two open arms (51 × 10 cm) and two enclosed arms (51 × 10 × 41 cm) extending from a common central platform (10 × 10 cm) that was elevated 55 cm above the floor on a central pedestal. The test was run at week 12. Animals were acclimatised to the testing room for 30 min prior experiment and subsequently placed in the centre of the maze facing an open arm to begin. Animal behaviour was videotaped for the duration of the 5 min test. Frequency of open and closed arms entries were counted and percentage time in open arms compared with total time.

2.4.2. Open field test

The open field test is used as a mechanism to assess anxiolytic effects of compounds (Seibenhener and Wooten, 2015). It was performed at week 13, as described previously (O'Mahony *et al.,* 2014). Animals were habituated to the testing room for 30 min prior experiment. Subsequently, each animal was placed in the centre of a brightly illuminated white open field arena (60 × 40 cm; 60 lux) and their activities videotaped for 10 min. All the trials were conducted between 0900 and 1300 h. The arena was cleaned with 70% ethanol to avoid cue smell between each trial. At the end of each trial, animals were returned to their cages. Distance moved, velocity, percentage of time spent in inner zone and frequency of inner zone entries were analysed as recorded using a tracking system (Ethovision XT 13, Noldus).

2.4.3. Forced swim test

The forced swim test (FST) is the most widely used model for predicting antidepressant activity in rodents, and increased immobility in this test is generally considered to reflect a state of behavioural despair (Porsolt *et al.,* 1978). The test was performed at 15 weeks to determine depression-like behaviour as previously described (Slattery and Cryan, 2012). Initially, rats were individually placed in a glass cylinders (H: 45 cm; D: 20 cm) filled with water to 30 cm depth at 24 ± 1 °C for a 15 min pre-test period. Subsequently, the rats were removed from the water, dried and placed in their home cage. Water in the cylinders was changed

between each trial. The following day, 24 h after the first exposure, the rats were placed in the water cylinders for 5 min and behaviours were videotaped from above for subsequent analysis. The 5 min session was scored using a time-sampling technique, whereby the predominant behaviour (climbing, swimming or immobility) in each 5 s period of the 300 s trial was recorded. Climbing behaviour consisted of upward-directed movements of the forepaws along the side of the cylinder. Swimming behaviour was defined as movement (usually horizontal) throughout the cylinder. The rat was considered immobile when the only activity observed was that which was required by the rat to keep its nose above water.

2.3. Analysis of biological markers

2.3.1. Plasma corticosterone determination

Collection of blood samples was performed as previously described (Pusceddu *et al.* 2015). Briefly, blood samples were collected during day one of swim stress, via tail incision at five different time points; immediately before (baseline) and, 15 min, 45 min, 75 min and 105 min after the test was finished. Approximately 200 µL of blood was collected in a tube containing 10 µL of EDTA 0.1 M to avoid coagulation. Blood plasma was obtained by centrifugation (3500 *g*, 4 °C, 15 min). Corticosterone levels were measured using the Corticosterone ELISA kit (Enzo) according to the manufacturer instructions, and absorbance signal was detected using a conventional plate reader (Synergy HT, Biotek).

2.3.2. Plasma BDNF measurement

Immediately after sacrifice, trunk blood was collected in EDTA Vacutainer tubes. Blood plasma was obtained by centrifugation (3500 *g*, 4 °C, 15 min). Protein levels of brain-derived neurotrophic factor (BDNF) were determined using an electrochemiluminescence multiplex system (MSD, Gaithersburg, MD, USA) according to the manufacturer protocol. MSD plates were read and analysed using the MSD QuickPlex SQ 120 Instrument

2.3.3. Monoamine determination

Monoamine (noradrenaline, serotonin, dopamine, 5-hydroxyindoleacetic acid and 3,4-dihydroxyphenylacetic acid) concentrations in the brain stem of rats were determined using high-performance liquid chromatography (HPLC) technique as described previously (Clarke *et*

al., 2013). Briefly, brain tissues were sonicated in 500 µL of chilled mobile phase spiked with 4 ng/40 µL of N-Methyl 5-HT (Sigma Chemical Co., UK) as internal standard. The mobile phase contained 0.1 M citric acid, 5.6 mM octane-1-sulphonicacid (Sigma), 0.1 M sodium dihydrogen phosphate, 0.01 mM EDTA (Alkem/Reagecon, Cork) and 9% (v/v) methanol (Alkem/Reagecon) and was adjusted to pH 2.8 using 4 N sodium hydroxide (Alkem/Reagecon). Samples were subsequently centrifuged (22,000 *g*, 4 ^{o}C, 15 min) and supernatant was separated and injected (40 µL) onto the HPLC system. The HPLC system consisted of a SCL 10-Avp system controller, LC-10AS pump, SIL-10A autoinjector (with sample cooler maintained at 40 ^{o}C), CTO-10A oven, LECD 6A electrochemical detector (Shimadzu) and an online Gastorr Degasser (ISS, UK). A reverse-phase column (Synergi 4u MAX-RP 80A, 250 4.6 mm, Phenomenex) maintained at 30 ^{o}C was employed in the separation (flow rate 2 mL min^{-1}), the glassy carbon working electrode combined with an Ag/AgCl reference electrode (Shimdazu) was operated at +0.8 V and the chromatograms generated were analysed using Class-VP 5 software (Shimadzu). Monoamines and monoamine metabolites were identified by their characteristic retention time as determined by standard injections which were run at regular intervals during the sample analysis. Analyte: internal standard peak height ratios were measured and compared with standard injections. Results were expressed as nanogram of neurotransmitter per gram fresh weight of tissue.

2.3.4. Brain fatty acid analysis

Fatty acid analysis was performed on the frontal cortex brain region. Lipids were extracted from tissues using chloroform: methanol (2:1 v/v) as previously described (Folch *et al.,* 1957). Lipid extracts were dried down under a flow of nitrogen at 50 °C. Dried samples were re-dissolved in 1 mL of hexane with internal standard tridecanoate (C19:0; 500 ppm). Sodium methoxide solution (2M) was added to the diluted samples to prepare the fatty acid methyl esters (FAME). Hydrochloric acid solution in methanol (2M) was added to neutralise the solution. Samples were divided between two GC glass vials and stored at -20 °C until analysis by gas chromatography. FAME separation was performed via GC (Agilent 7890B, Agilent Technologies, Santa Clara, CA, USA) with flame ionisation detection (Chrompack CP Sil 88 column, Chrompack, JVA Analytical, 100 m, 0.25 mm internal diameter, 0.25 µm film thickness) and helium as the carrier gas. The GC column oven cycle was programmed initially

at 80 °C for 8 min followed by an increase by 8.5 °C/min to a final column temperature of 200 °C. Sample injection (0.5 µL) was automated (CTC Autosampler, Model 80) on an Agilent Multimode Inlet on-column temperature programmable injector. Peak integration was performed using the Agilent OpenLAB Chemstation software (Version A.01.06.111). Identification of individual FAME was achieved by comparison of retention times with pure FAME standards (Nu-Chek Prep, Elysian, MN, USA) and quantification against an internal standard (C19:0). Results are expressed as g 100 g^{-1} of total FAME.

2.5. Microbial analysis

2.5.1. DNA extraction & 16S rRNA sequencing

DNA extraction from frozen faecal samples was performed using a QIAGEN QIAamp Fast DNA Stool Mini Kit (Qiagen Ltd, Manchester, England) according to the manufacturer's protocol. DNA was quantified by NanoDrop spectrometry. The V3-V4 variable region of the 16S rRNA gene was amplified from the DNA extracts using the Illumina 16S metagenomic sequencing library protocol. The PCR reactions were performed in a 25 µL reaction volume containing DNA template, 12.5 µL 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), 5 µl each of forward and reverse primers (1 µM), and PCR grade water to final volume. PCR amplification conditions included initial denaturation at 95 °C for 5 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 5 min. PCR products were cleaned using AMPure XP magnetic bead based purification (Labplan, Dublin, Ireland). This was followed by indexing PCR which attached Nextera XT barcodes and Illumina sequencing adapters to the 5' overhangs and another round of AMPure XP clean-up. After quantifying the samples, using Invitrogen Qubit 4 Fluorometer and high sensitivity DNA quantification assay kit (BioSciences, Dublin, Ireland), they were pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. Samples were sequenced on the MiSeq sequencing platform at the Teagasc Sequencing Facility, using a 2 x 250 bp cycle kit, following standard Illumina sequencing protocols.

2.5.2. Bioinformatics

Two hundred and fifty base pair paired-end reads were assembled using FLASH. The QIIME suite of tools, v1.8.0, was used for further processing of paired-end reads, including quality filtering based on a quality score of >25 and removal of mismatched barcodes and sequences below length thresholds (Caporaso *et al.* 2010). De-noising, chimera detection and operational taxonomic unit (OTU) grouping at 98% similarity were performed using USEARCH v7 (64-bit) (Edgar 2010). Taxonomic ranks were assigned by alignment of OTUs using PyNAST to the SILVA SSURef database release 128 (Quast *et al.* 2012). Alpha and beta diversities were calculated using QIIME on weighted Unifrac distance matrices.

16S microbiota data was entered into Calypso (Zakrzewski *et al.* 2016) for further analysis and statistical testing. Principal co-ordinate analysis (PCoA) plots were visualised using Bray-Curtis calculated distances and differences between dietary treatments were calculated using permutational MANOVA (Adonis) analysis. The Benjamini-Hochberg adjustment procedure was applied with the false discovery rate (FDR) set at 20% to correct for multiple testing.

2.5.3. SCFA analysis

Caecal content samples were vortex-mixed with Milli-Q water (1:10 w/v), left to stand for 10 min at room temperature and centrifuged (10,000 *g*, 5 min) to pellet solids. The supernatant was filtered (0.2 µm) before transferring to a GC glass vial and 2-ethylbutyric acid (Sigma) was added as internal standard. SCFA concentrations were measured using a Varian 3800 GC flame-ionization system, fitted with a ZB-FFAP column (30 m 60.32 mm 60.25 mm; Phenomenex). Helium was used as the carrier gas at a flow rate of 1.3 mL min^{-1}. The initial oven temperature was set at 100 ^{o}C for 0.5 min, raised to 180 ^{o}C at 8 ^{o}C min^{-1} and held for 1 min, then increased to 200 ^{o}C at 20 ^{o}C min^{-1}, and finally held at 200 ^{o}C for 5 min. The temperature of the injector and the detector were set at 240 ^{o}C and 250 ^{o}C, respectively. A standard curve made from a standard mix of acetic acid, valeric acid, propionic acid, iso-propionic acid, n-butyric acid and iso-butyric acid (Sigma) at seven concentrations. Peaks were integrated by using the Varian Star Chromatography Workstation version 6.0 software. Standards were included in each run to maintain the calibration.

2.6. Statistical analysis

Statistical analyses were performed using Microsoft Excel® and the SPSS® computer programs (SPSS Statistical Software, Inc., Chicago, IL.). Results are presented as mean ± standard deviation (SD). Differences between non-separated and maternally separated (MS) control groups were assessed by t-test analysis. Following this, differences between untreated and treated maternally separated groups were assessed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Non-parametric data from 16S sequencing analyses were subject to Kruskal-Wallis tests followed by Mann-Whitney tests. Significance level was determined at the 95% probability level. GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used for the generation of figures.

3. RESULT

The bodyweights of the rats in the five experimental groups were recorded from week six, two weeks before dietary intervention began. At week six, the average weight ranged from 150.42 ± 25.97 g for the NS-Con group to 172.92 ± 13.88 g for the MS-Con group. The animals weighed between an average of 348.33 ± 32.60 g for the NS-Con group and 380.83 ± 25.33 g for the MS-Con group at week 15. There was no significant difference in weights of animals in the five groups throughout this period (data not shown).

3.1. Behaviour tests

3.1.1. Elevated plus maze

No statistically significant differences were found between the amount of time spent or the number of entries that NS and MS animals carried out in the open and closed arms during the elevated plus maze test (Figure S7.1.).

3.1.2. Open field test

Anxiety-like behaviour was represented as a function of locomotion in the open field arena. Animals that underwent maternal separation spent less time in the inner zone ($t(21.00) = 2.156$, $p < 0.05$) and entered this area less times ($t(21.00) = 2.868$, $p < 0.01$) compared to animals that had not undergone the maternal separation procedure. These MS-induced increases in anxiety-like behaviour were not recorded in animals that underwent dietary treatment with fish oil, fluoxetine or the combination of fish oil and fluoxetine (Figure 7.2a. and b.). No difference was found between the distance travelled or speed of movement of the animals in the two control groups or between the non-treated and dietary treated MS groups, distinguishing the genuine effects on anxiety behaviour from a sedative or hyperactive effect (Figure 7.2c. and d.).

3.1.3 Forced swim test

Depression-like behaviour is represented as a function of immobility time. Maternally separated control animals swam significantly less ($t(19.84) = 2.611$, $p < 0.05$) and spent significantly more time immobile compared to non-separated animals ($t(21.96) = 2.349$, $p <$

0.05; Figure 7.2d.). Animals fed a diet supplemented with fluoxetine swam significantly more, and hence were significantly less immobile, compared to the MS control animals ($F(3, 41) = 10.41$, $p < 0.001$). These behaviours were not maintained in animals fed fluoxetine in conjunction with fish oil. No significant differences in climbing behaviour was recorded between any groups.

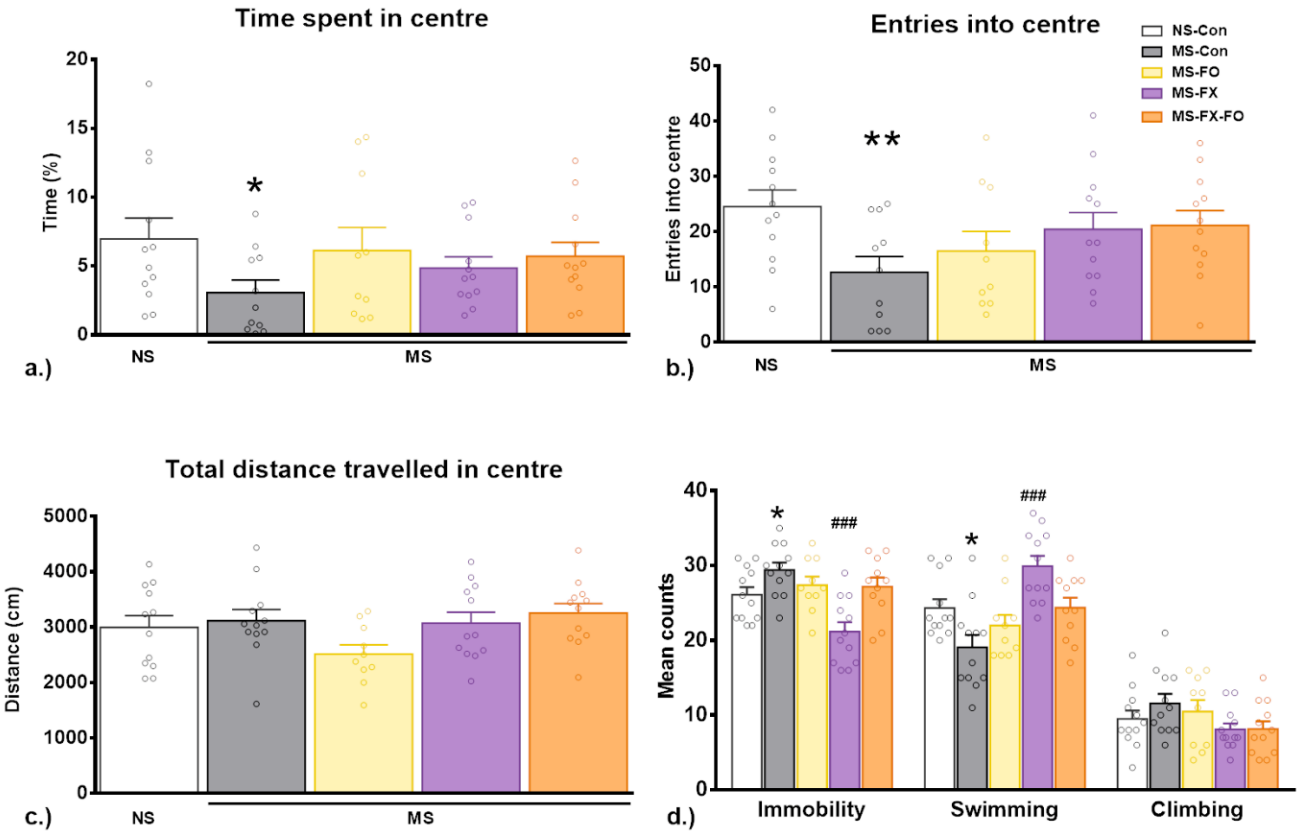

Figure 7.2. Open field test: percentage of time spent in open area (a.), total number of entries into open area (b.), total distance travelled into open area (c.) during open field test, and forced swim test: mean number of 5 s periods within the 300-s trial spent immobile, swimming of climbing (d.). Values are means + SD for each experimental group (NS-Con, MS-Con, MS-FX and MS-FO-FX (n=12) and MS-FO (n=10)). Significantly different from the non-separated control rats: * (t-test; $p < 0.05$), ** (t-test; $p < 0.01$).

3.2. Biological markers

3.2.1. Plasma corticosterone and BDNF

Plasma was analysed for corticosterone and BDNF. Corticosterone levels increased substantially in the first 30 min, when the animals underwent the swim stress, after which it decreased. The non-separated and maternally separated control groups did not differ significantly. However, at the 30 min sampling point, plasma corticosterone levels were significantly lower in animals from both fluoxetine treated groups compared to the non-treated MS animals (Figure 7.3a.). Dietary treatment with fish oil, fluoxetine, and a combination of fluoxetine and fish oil, all resulted in significantly lower levels of overall corticosterone levels (area under the curve; AUC) compared to the MS control animals ($F(3, 41) = 4.508$, $p < 0.05$; Figure 7.3b.). No differences in BDNF plasma concentrations were found in animals from the five experimental groups (data not shown).

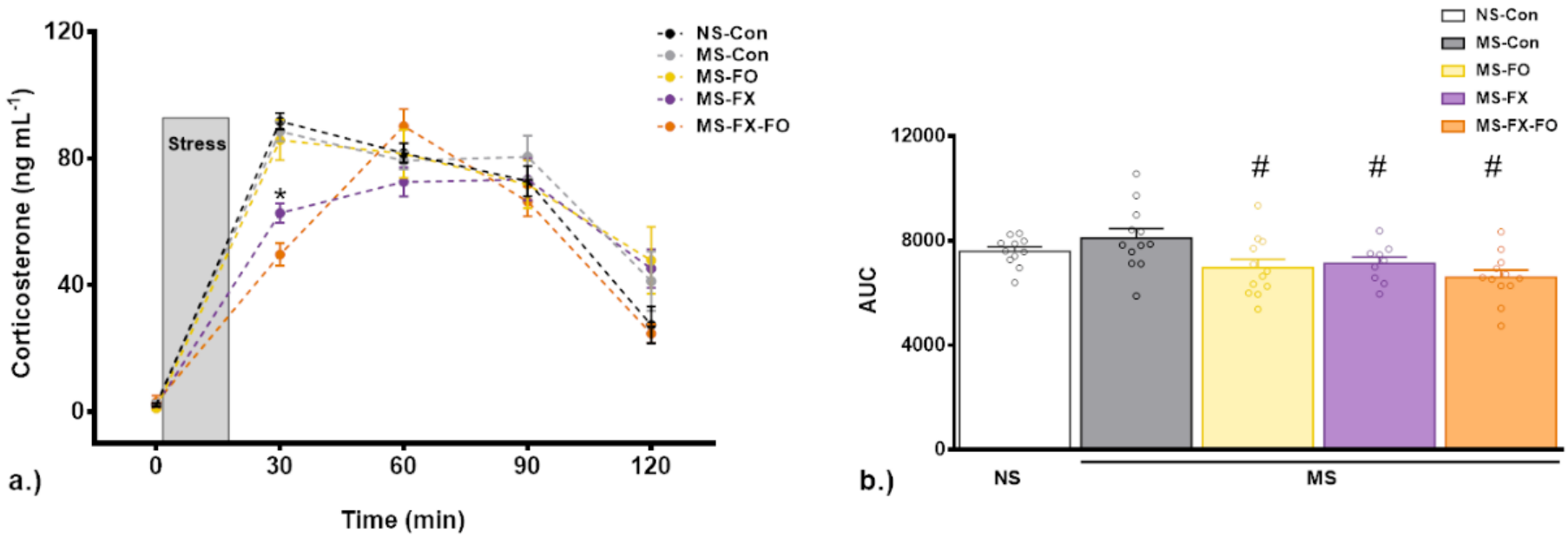

Figure 7.3. Plasma corticosterone levels analysed from blood samples collected during day one of the forced swim test, at five time points; immediately before the swimming (baseline), 15 min, 45 min, 75 min and 105 min after the test was finished (a.) and area under the curve (AUC) values (b.) for each experimental group (NS-Con, MS-Con, MS-FX and MS-FO-FX (n=12) and MS-FO (n=10)). Significantly different from the maternally separated control rats, # (one-way ANOVA; $p < 0.05$).

3.2.2. Monoamine determination

Concentrations of monoamines and their metabolites in the brainstem tissue of rats were measured (Table 7.1.). Non-separated animals were found to have the highest levels of DA, whereas NA and 5-HT were highest in maternally separated animals who received the fish oil treatment. The 5-HT metabolite, 5-HIAA, was highest in non-separated animals, while the DA metabolite DOPAC was highest in maternally separated animals who received the fluoxetine treatment. The maternally-separated control group had significantly lower 5-HIAA ($t(18.28) = 4.160$, $p < 0.001$), and hence significantly lower 5-HIAA/5-HT ratio compared to the non-separated group ($t(13.03) = 3.406$, $p < 0.01$). Moreover, dietary treatment with fish oil, fluoxetine, and a combination of fluoxetine and fish oil, all resulted in significantly lower levels of 5-HIAA ($F(3, 42) = 18.36$, $p < 0.001$), and hence significantly lower 5-HIAA/5-HT ratio ($F(3, 42) = 5.755$, $p < 0.01$) in maternally separated animals compared to the MS control animals.

Table 7.1. Monoamine concentrations detected in brain stem tissue of rats from different experimental groups (NS-Con, MS-Con, MS-FX and MS-FO-FX (*n*=12) and MS-FO (*n*=10)). 5-HT = serotonin, 5-HIAA = 5-hydroxyindoleacetic acid, 5-HIAA/5-HT = ratio of 5-hydroxyindoleacetic acid to serotonin, NA = noradrenaline, DA = dopamine, DOPAC = 3,4-dihydroxyphenylacetic acid, DOPAC/DA = ratio of 3,4-dihydroxyphenylacetic acid to dopamine. Values are means ± SD. Significantly different from the non-separated control rats: * (t-test; $p < 0.05$), ** (t-test; $p < 0.01$), *** (t-test; $p < 0.001$). Significantly different from the maternally separated control rats: $^{\#}$ (one-way ANOVA; $p < 0.05$), $^{\#\#}$ (one-way ANOVA; $p < 0.01$), $^{\#\#\#}$ (one-way ANOVA; $p < 0.001$).

	NS-Con	MS-Con	MS-FO	MS-FX	MS-FO-FX
5-HT	607.90 ± 88.12	604.12 ± 78.69	669.82 ± 61.60	516.72 ± 67.78	640.06 ± 128.01
5-HIAA	774.98 ± 104.53	606.83 ± 77.15***	474.86 ± 39.74$^{\#\#\#}$	387.21 ± 61.11$^{\#\#\#}$	473.86 ± 88.11$^{\#\#\#}$
5-HIAA/5-HT	1.29 ± 0.24	1.01 ± 0.10**	0.71 ± 0.08$^{\#\#}$	0.76 ± 0.15$^{\#\#}$	0.79 ± 0.29$^{\#}$
NA	428.00 ± 51.33	460.03 ± 91.38	521.35 ± 59.55	447.64 ± 60.48	349.57 ± 61.55$^{\#\#}$
DA	69.61 ± 6.92	56.00 ± 23.31	49.52 ± 5.42	37.07 ± 6.63$^{\#\#}$	55.72 ± 11.01
DOPAC	27.48 ± 9.69	26.41 ± 8.46	23.79 ± 4.13	30.83 ± 11.60	22.50 ± 5.20
DOPAC/DA	0.39 ± 0.15	0.58 ± 0.26*	0.48 ± 0.06	0.76 ± 0.24	0.41 ± 0.09

3.3.3. Brain fatty acid analysis

Administration of diets supplemented with fish oil and fluoxetine elicited significantly different fatty acid compositions in the frontal cortex of the rats (Table 7.2.). The non-separated and maternally separated groups did not show any statistically significant difference in the fatty acid composition of their frontal cortex. DHA comprised 16 – 18% of the detected fatty acids in all groups. The two groups whose diets were supplemented with fish oil had significantly higher relative abundances compared to the other groups (two-way ANOVA; $F(25, 1378) = 1663$, $p < 0.05$). The animals treated with fish oil and fluoxetine had significantly more total PUFA and n-3 PUFA compared to the non-separated animals ($F(4, 53) = 3.771$, $p < 0.05$), while animals treated with fish oil only had a significantly lower n-3 : n-6 PUFA ratio compared to the non-separated animals ($F(4, 53) = 4.038$, $p < 0.05$).

Table 7.2. Fatty acid composition of frontal cortex brain region of rats from different experimental groups (NS-Con, MS-Con, MS-FX and MS-FO-FX (*n*=12) and MS-FO (*n*=10)). Values are means ± SD. Groups with significantly different relative abundances of fatty acids are denoted with a different letter (ANOVA, $p < 0.05$).

	NS-Con	MS-Con	MS-FO	MS-FX	MS-FO-FX
SFA					
C14:0	0.06 ± 0.14	0.01 ± 0.03	0.07 ± 0.07	0.05 ± 0.11	0.03 ± 0.07
C16:0	24.73 ± 0.47	24.20 ± 0.85	24.54 ± 0.38	24.54 ± 0.53	24.60 ± 0.64
C18:0	22.44 ± 0.48^{a}	22.34 ± 0.52^{a}	22.12 ± 0.33^{a}	22.12 ± 0.58^{a}	21.03 ± 3.10^{b}
C20:0	0	0	0	0	0.03 ± 0.10
C22:0	0.27 ± 0.05	0.24 ± 0.08	0.28 ± 0.03	0.28 ± 0.04	0.29 ± 0.06
C24:0	0.43 ± 0.04	0.47 ± 0.21	0.39 ± 0.03	0.39 ± 0.04	0.38 ± 0.08
Total SFA	47.87 ± 0.79	47.27 ± 1.00	47.31 ± 0.54	47.38 ± 1.08	46.36 ± 3.46
MUFA					
C16:1 n-7	0.38 ± 0.03^{a}	0.34 ± 0.12^{a}	0.52 ± 0.10	0.36 ± 0.05^{a}	1.49 ± 3.23^{b}
C18:1 n-9	18.87 ± 0.45^{ab}	19.06 ± 1.08^{ab}	19.41 ± 0.70^{ac}	18.36 ± 0.58^{b}	19.76 ± 0.75^{c}
C18:1 n-7	3.74 ± 0.17	3.82 ± 0.26	4.01 ± 0.21	3.75 ± 0.11	4.36 ± 1.95
C20:1 n-9	0	0	0.01 ± 0.01	0	0
C22:1 n-9	6.08 ± 0.21^{a}	6.04 ± 0.19^{a}	5.60 ± 0.32^{ab}	6.08 ± 0.19^{a}	4.94 ± 0.72^{b}
C24:1 n-9	3.74 ± 0.18^{a}	3.70 ± 0.16^{a}	3.16 ± 0.25^{ab}	3.63 ± 0.11^{a}	2.62 ± 0.35^{b}
Total MUFA	32.8 ± 0.72	32.97 ± 1.27	32.71 ± 0.59	32.19 ± 0.87	33.17 ± 4.29
PUFA					
n-6 PUFA					
C18:2 n-6	0.91 ± 0.07	0.85 ± 0.10	0.37 ± 0.27	0.92 ± 0.05	0.53 ± 0.66
C18:3 n-6	0.02 ± 0.02	0.02 ± 0.02	0.02 ± 0.02	0.01 ± 0.02	0.02 ± 0.02
C20:2 n-6	0.66 ± 1.08	0.88 ± 1.26	0.36 ± 0.46	0.98 ± 1.57	0.28 ± 0.52
C20:3 n-6	0.16 ± 0.02	0.16 ± 0.02	0.14 ± 0.02	0.13 ± 0.03	0.14 ± 0.04
C20:4 n-6	0	0.01 ± 0.01	0.02 ± 0.01	0	0.23 ± 0.72
C22:2 n-6	0.27 ± 0.38	0.42 ± 0.64	0.19 ± 0.24	0.40 ± 0.81	0.13 ± 0.25
C22:4 n-6	0.03 ± 0.03	0.02 ± 0.02	0.02 ± 0.01	0.02 ± 0.02	0.14 ± 0.38
Total n-6 PUFA	2.05 ± 1.34	2.35 ± 1.86	1.12 ± 0.69	2.46 ± 2.31	1.47 ± 2.03
n-3 PUFA					
C18:3 n-3	0.98 ± 0.08	1.05 ± 0.21	1.04 ± 0.13	0.94 ± 0.11	0.95 ± 0.19
C20:5 n-3	0.01 ± 0.03	0.03 ± 0.09	0.02 ± 0.02	0.05 ± 0.12	0.05 ± 0.03
C22:5 n-3	0.08 ± 0.03	0.07 ± 0.03	0.12 ± 0.08	0.07 ± 0.04	0.35 ± 0.12
C22:6 n-3	16.13 ± 0.31^{a}	16.27 ± 0.92^{ac}	17.68 ± 0.40^{b}	16.90 ± 0.79^{c}	17.65 ± 2.70^{b}
Total n-3 PUFA	17.21 ± 0.32^{a}	17.41 ± 0.76^{ab}	18.86 ± 0.33^{ab}	17.97 ± 0.72^{ab}	19.00 ± 2.79^{b}
Total PUFA	19.27 ± 1.31^{a}	19.76 ± 1.82^{ab}	19.98 ± 0.69^{ab}	20.43 ± 1.79^{ab}	20.47 ± 1.32^{b}
n-6: n-3	0.12 ± 0.06^{a}	0.14 ± 0.08^{ab}	0.06 ± 0.03^{b}	0.14 ± 0.09^{ab}	0.08 ± 0.11^{ab}

3.3. Microbial analysis

3.3.1. 16S gut microbiota

The gut microbiota compositions of the animals were analysed by extracting and sequencing 16S rDNA from faecal pellets removed from the lower intestines during dissection. After quality filtering, reads per sample ranged from 180,059 – 2,884,732. Joining efficiency was 94.17 ± 8.13% and after sequence clustering 9,371 individual OTUs were identified. The α – diversity of the groups was compared using a range of indices (Shannon, Chao1, Richness, PD-wholetree and Simpson). Differences were found to be statistically insignificant in all tests (Figure 7.4a. and b.). Although, when using the Chao1 index, NS-Con had the lowest diversity (1713 ± 517) and MS-FO animals had the highest diversity (2044 ± 147) and after one-way ANOVA ($F(4, 51) = 2.5$). $p = 0.054$ (Figure 7.4b.).

The β–diversity of the groups was visualised using a PCoA Bray Curtis plot and differences were calculated using permutational multivariate ANOVA ('Adonis') analysis (Figure 7.5a., c. and e.). Significant separation of the gut microbial compositions by treatment was found at phylum, family and genus level (PERMANOVA, $R^2 = 0.195$, $p < 0.001$). The two treatment groups on diets containing fish oil clearly separated away from the other treatment groups.

This result was supported by differences in relative abundance of microbiota. The untreated, maternally separated groups shared the greatest number of OTUs with the non-separated group, and of these only 12 OTUs were found at a significantly different relative abundance. Similarly, MS-FO and MS-FX-FO animals shared a similar number of OTUs which included only 13 OTUs at a significantly different relative abundance, whereas MS-FO animals, while sharing similar numbers of OTUs, differed significantly more, in terms of relative abundance, from NS-Con and MS-Con animals (by 41 and 31 OTUs, respectively). MS-FX-FO animals also had a large number of OTUs at significantly different relative abundances to NS-Con and MS-Con animals (34 and 27 OTUs, respectively). Interestingly, 23 OTUs were detected at significantly different relative abundances in MS-FX and MS-FX-FO animals. This indicates that fish oil was the principal treatment eliciting changes in faecal microbial relative abundance.

At phylum level, all experimental groups were dominated by Firmicutes and Bacteroidetes (94.2 – 97.6%; Figure 7.5b.). The two groups on the control diet did not have

any significant differences at this level. Animals supplemented with fish oil had higher levels of Bacteroidetes and Deferribacteres and lower levels of Saccharibacteria, Tenericutes and Firmicutes. However, the difference in Bacteroidetes ratio became insignificant after implementing the false discovery rate. Animals supplemented with only fluoxetine also had lower levels of Saccharibacteria compared to animals on the control diet.

The dominant families in all the experimental groups were *Lachnospiraceae, Bacteroidales* S24-7 group, *Ruminococcaceae* and *Bacteroidaceae* (Figure 7.5d.). MS-Con animals had lower relative abundances for many microbial families compared to NS-Con animals. These differences were found to be statistically significant for *Micrococcaceae, Caldicoprobacteraceae, Christensenellaceae* and *Streptococcaceae.* Administration of fluoxetine on its own increased the relative abundance of these families, however, the levels were still statistically lower than those found in samples from NS-Con animals. Supplementation with fish oil did not cause the same widespread increases. However, the family *Nocardiaceae* was at a significantly higher relative abundance in the two fish oil treated groups compared to the MS-Con group, while *Prevotellaceae* was also at a notably higher level and this difference was statistically significant for the MS-FX-FO group.

Of 199 genera detected, 143 were shared by all experimental groups. The dominant genera in all experimental groups were *Bacteroidales* S24-7 group bacterium 1, *Lachnospiraceae* NK4A136 group*, Bacteroidales* S24-7 group bacterium 2, *Bacteroides* and *Ruminococcaceae* bacterium. The groups varied significantly in the relative abundance of 67 of these. Similar to family level, MS-Con animals had lower relative abundance of the majority of these genera compared to NS-Con animals. MS-Con animals had significantly lower *Rothia, Caldicoprobacter, Lachnospiraceae_NC2004_group, Ruminococcaceae_UCG011* and *Ruminococcus_2* and significantly higher Mollicutes bacterium, *Ruminiclostridium* 6 and *Parabacteroides*, as well as two other unidentified bacteria, compared to NS-Con animals (Figure 7.5f.).

Animals supplemented with only fluoxetine had significantly lower abundance of a Mollicutes bacterium, *Candidatus Saccharimonas* and *Ruminiclostridium* and higher relative abundance of *Neochlamydia, Lachnoclostridium*, *Acetitomaculum* and *Stenotrophomonas* compared to MS-Con animals. *Acetitomaculum* and *Stenotrophomonas* were also significantly higher compared to NS-Con animals. The MS-FO group had 25 genera at significantly lower ratios compared to the MS-Con group, 13 of which were also significantly

reduced in the MS-FX-FO group. The majority of these genera are associated with SCFA production, often in particular butyrate production. The MS-FO and MS-FX-FO groups had seven and 12 genera at significantly higher relative abundances compared to MS-Con animals, respectively. These genera were; *Rhodococcus, Enterococcus, Shuttleworthia, Ruminiclostridium_9, Parabacteroides, Thalassospira, Bacillus* and *Propionibacterium*, and *Eisenbergiella, Alloprevotella, Mucispirillum, Tyzzerella, Bilophila, Alistipes, Gordonibacter, Rikenellaceae_RC9_gut_group, Lachnospiraceae_UCG004* and *Odoribacter*, respectively.

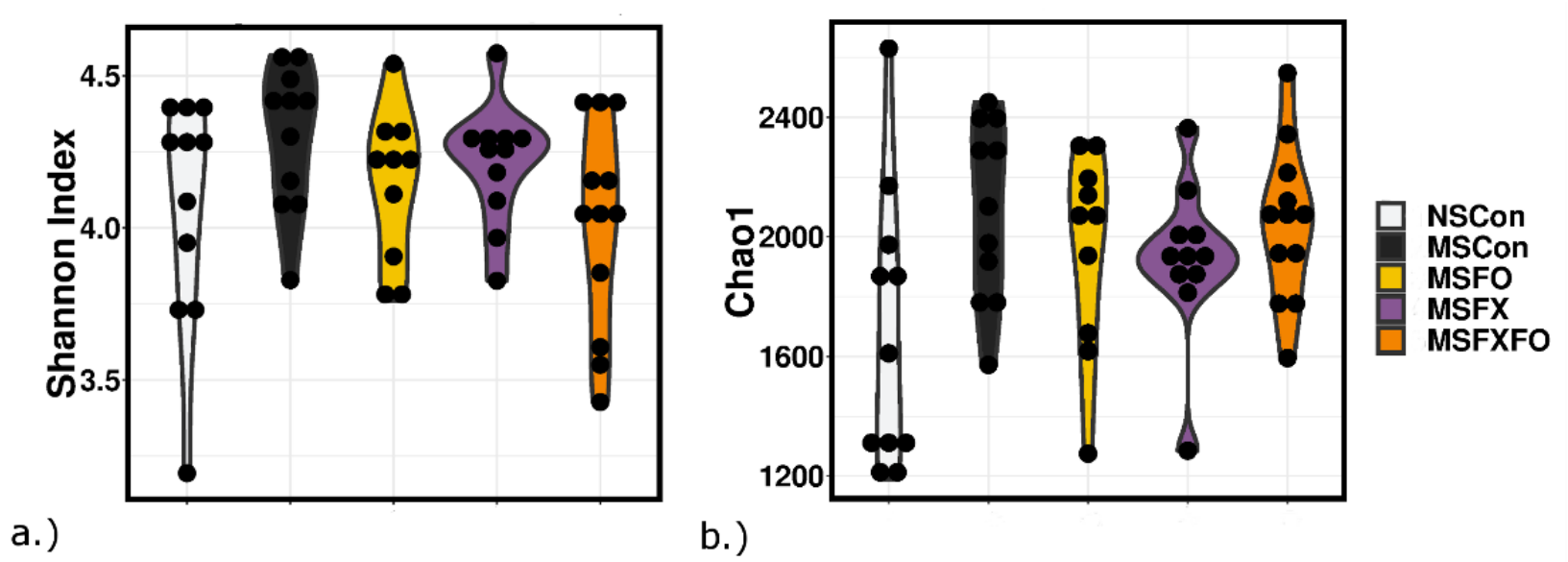

Figure 7.4. The Shannon (a.), and Chao1 (b.) α-diversity measures of 16S rRNA microbiota gut community compositions in faecal microbiomes of rats from different experimental groups (NS-Con, MS-Con, MS-FX and MS-FO-FX (*n*=12) and MS-FO (*n*=10)).

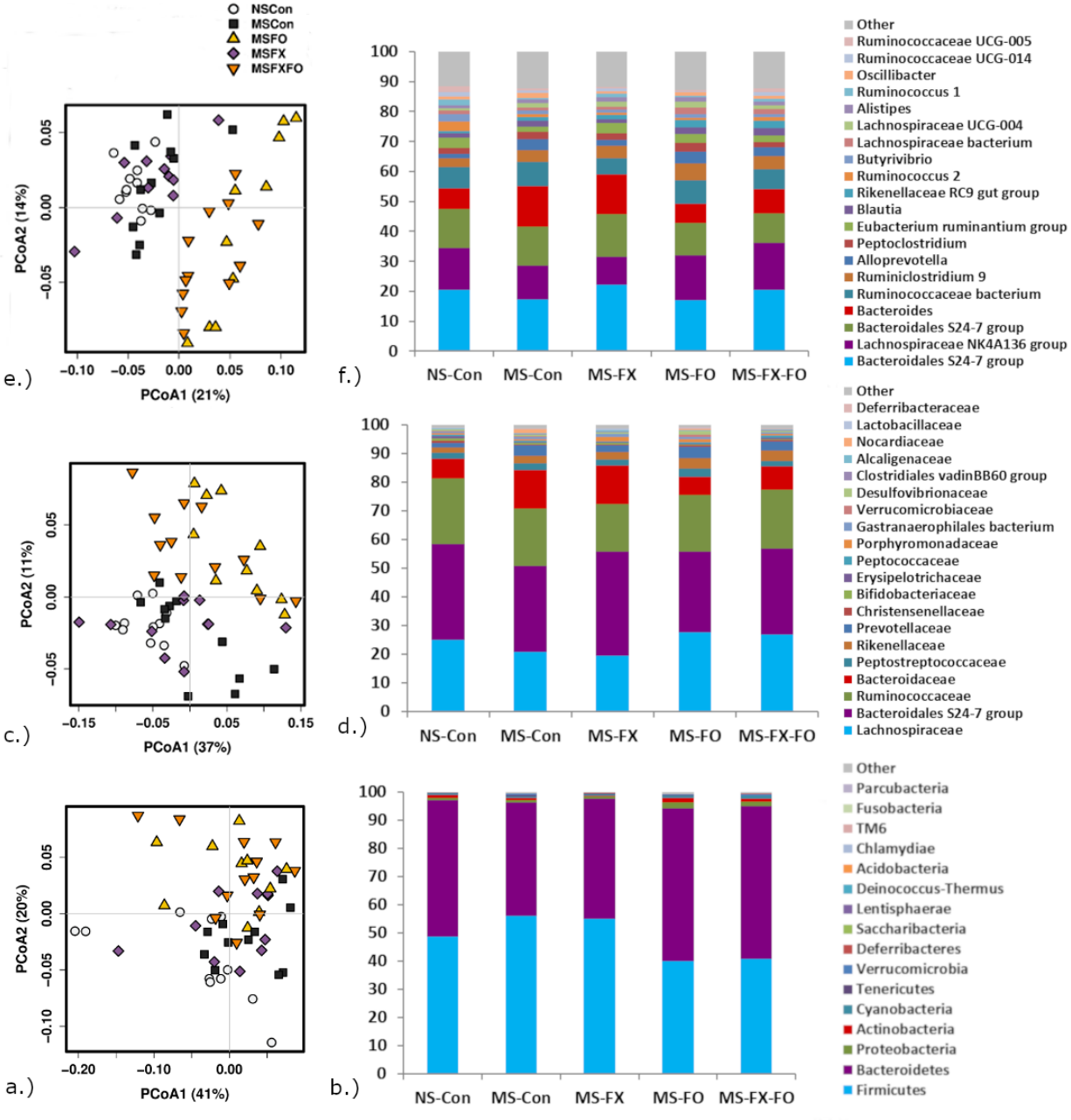

Figure 7.5. 16S rRNA sequencing analysis. PCoA ordination of Bray-Curtis distances and relative abundance of top 20 most abundant OTUs, respectively, at the phylum (a. & b.), family (c. & d.), and genus (e. & f.) taxonomic ranks of bacterial taxa associated with treatment groups; NSCon (○): non-separated control, MSCon (■): maternally separated control. MSFO (△): maternally separated with fish oil diet, MSFX (◆): maternally separated with fluoxetine diet, MSFOFX (▼): maternally separated with fish oil and fluoxetine in diet.

3.3.2. SCFA analysis

The concentration of total short-chain fatty acids (SCFA) were significantly lower in maternally separated animals compared to non-separated animals (t (19.88) = 2.451, $p < 0.05$; Figure 7.6a.). Maternally separated animals treated with fluoxetine had significantly higher concentrations compared to the MS-Con animals (F (3, 42) = 10.01, $p < 0.05$). These results were mirrored when measuring acetate on its own. In addition, maternally separated animals treated with fluoxetine and fish oil had significantly lower concentrations of acetate compared to the MS-Con animals (F (3, 42) = 10.38, $p < 0.05$; Figure 7.6b.). MS-Con animals also had significantly lower levels of propionate, compared to NS-Con animals and this SCFA was also found to be at significantly higher concentrations in MS-FX animals compared to MS-Con animals (Figure 7.6c.). Butyrate was detected at significantly lower concentrations in both groups treated with fish oil compared to the MS-Con group (F (3, 42) = 9.687, $p < 0.05$; Figure 7.6d.). No significant difference in valerate levels was found between any of the groups (Figure 7.6e.). Branched short chain fatty acids (BCFA) were at significantly lower levels in the MS-Con group compared to the NS-Con group (t (17.38) = 2.385, $p < 0.05$). Dietary treatment did not create significant differences in levels of BCFA between the maternally separated groups (Figures 7.6f., g. and h.).

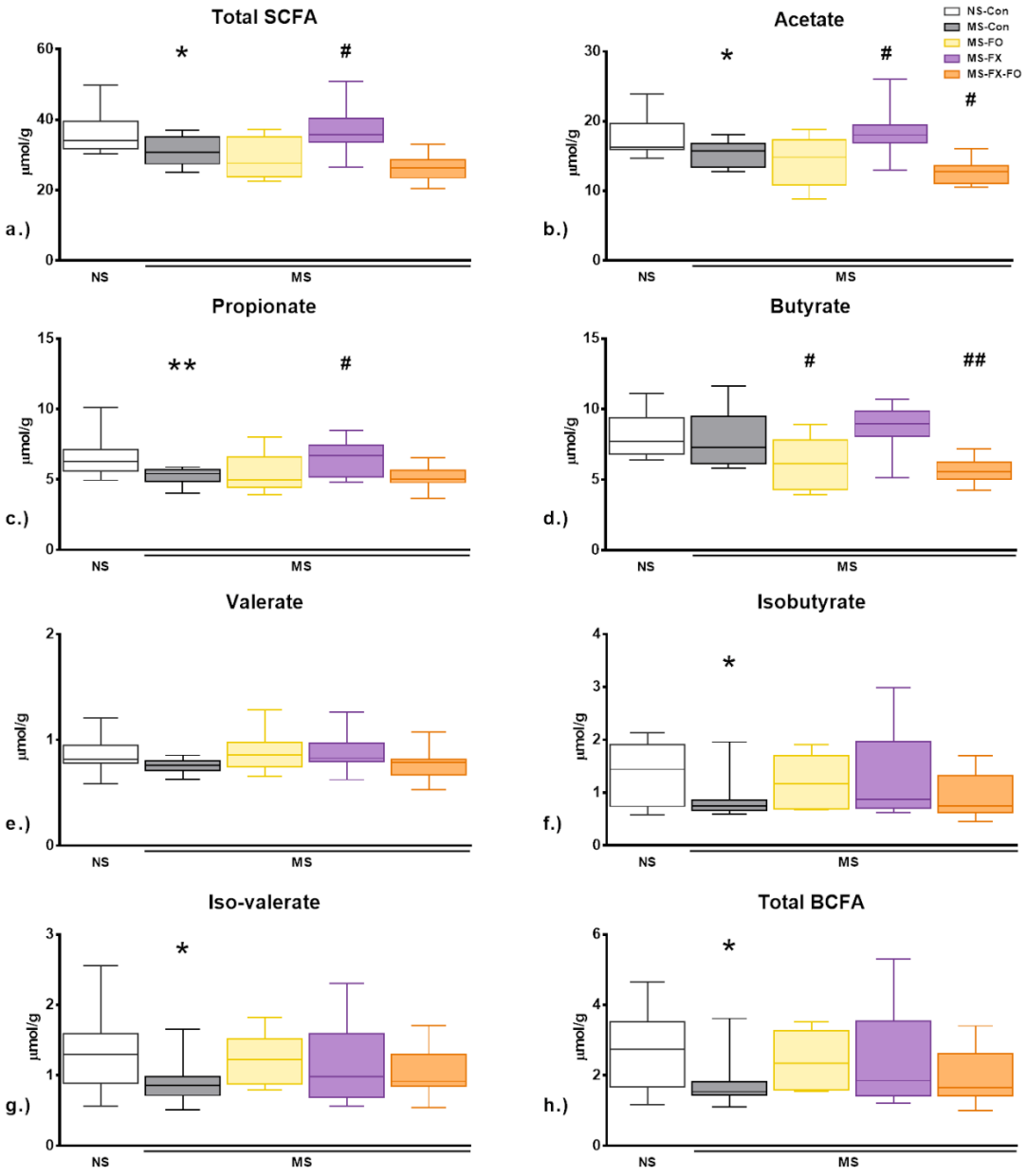

Figure 7.6. Short chain fatty acids (µmol/g wet weight): total SCFA (a.), acetate (b.), propionate (c.), butyrate (d.), valerate (e.), iso-butyrate (f.), iso-valerate (g.) and total BCFA (h.) detected in caecum contents of rats for each experimental group (NS-Con, MS-Con, MS-FX and MS-FO-FX (n=12) and MS-FO (n=10)). Significantly different from the non-separated control rats: * (t-test; $p < 0.05$) and significantly different from the maternally separated control rats, # (t-test; $p < 0.05$).

4. DISCUSSION

SSRIs, such as fluoxetine, are the first-line pharmacological treatment for depression. However, treatment-resistant depression is common, with 15% of patients achieving only partial response, whilst non-response is present in 19 – 34% (Vieta and Colom 2011). Thus, continued development of alternative and adjunct therapies is important. Diet is intrinsically linked with health and development in the understanding of the microbiota – gut –brain axis has provided potential mechanistic pathways for harnessing the benefits of dietary nutrients for treatment of mental illnesses. In this study, we have shown that the maternal separation animal model of early life stress induces a depressive-like phenotype in male Sprague Dawley rats. This model targets early developmental periods when the brain displays enhanced plasticity and is vulnerable to environmental perturbations. It often results in enhanced anxiety (Mathieu *et al.* 2011; Zhang *et al.* 2012) and depression-like (Réus *et al.* 2011; Leussis *et al.* 2012) behaviour and impaired spatial learning and memory (Wang *et al.* 2011; Sousa *et al.* 2014) in later life. The specific neurobiological changes remain to be fully characterised but alterations within the brain at a cellular level, with altered neuroendocrine activity, immune function and neurotransmission have been reported in previous animal studies (Harrison and Baune 2014). As expected, we show that maternal separation resulted in behaviours indicative of depressive- and anxiety-like symptoms, as well as increased stress-induced plasma corticosterone levels and alterations in brain stem neurotransmitter metabolism. Many of the neurobiological and behavioural symptoms were reduced or blocked when the MS animals were treated with a diet supplemented with fish oil or fluoxetine from eight weeks of age onwards.

Hyperactivity of the HPA axis is one of the most frequently cited perturbations associated with early life stresses and maternal separation models (Heim *et al.* 2000; O'Mahony *et al.* 2011). Dysfunction of the HPA system increases glucocorticoid and corticotropin releasing factor levels in the hippocampus, resulting in neuronal degeneration, reduced neurogenesis and reduced neuroplasticity, causing a failure in the negative feedback of the HPA axis and ultimately decreased hippocampus volume, associated with major depressive disorder (MDD; Mahar *et al.* 2014; Boku *et al.* 2018). High levels of plasma corticosterone in maternally separated animals in this study indicates HPA system dysfunction. However, MS animals who received dietary supplementation with fish oil,

fluoxetine and a combination of fish oil and fluoxetine all had significantly lower plasma corticosterone levels compared to untreated MS animals. Omega-3 PUFA, in particular DHA, are essential elements of neural cell membranes. They play an important role in neurogenesis, synaptogenesis, neuronal differentiation, neurite outgrowth and maintenance of membrane fluidity (Romano *et al.* 2017). Their structural and regenerative characteristics may impede initial effects of stress-induced increases in glucocorticoid levels and help maintain a balanced negative feedback system within the HPA axis. Fluoxetine has frequently been reported to lower plasma corticosterone/cortisol levels, indicating its influence on the HPA system. Previous studies investigating potential mechanisms have found it decreases corticotropin-releasing hormone mRNA levels and increases the level of mineral-corticoid receptors in the hypothalamus (Brady *et al.* 1992). It has also been found to alter gene expression relating to glucocorticoid receptors and G protein receptor coupling in the brain (David *et al.* 2009; Heydendael and Jacobson 2010).

Treatment of MS animals with fish oil, fluoxetine and a combination of the two also significantly altered monoamine levels in the brain stem. All three treatments significantly reduced serotonin turnover, and hence 5-HIAA levels, compared to untreated MS animals. It has long been thought that a deficiency of brain monoaminergic activity occurs in depressive states (Elhwuegi 2004). However, studies have reported varying and sometimes contradictory findings of the monoamine-related neurochemistry and architecture observed between depressed and control groups. Previously, an accumulation of studies associated reduced levels of 5-HIAA in cerebrospinal fluid with affective disorders, in particular aggressive behaviours and suicides (Bortolato *et al.* 2010). However, it is now known that concentrations of 5-HIAA in cerebrospinal fluid can be affected by numerous peripheral factors (Cryan and Leonard 2000). Moreover, a recent meta-analysis found that the evidence for low cerebrospinal fluid 5-HIAA being related to the pathophysiology of MDD is poor (Pech *et al.* 2018). In line with our results, studies investigating the effects of chronic fluoxetine administration in rats undergoing forced swim tests have reported reduced 5-HIAA and serotonin turnover compared to untreated animals (Shishkina *et al.* 2012; McNamara *et al.* 2013). Such findings are supported by *in-vitro* and *in-vivo* studies that have shown fluoxetine to work as an inhibitor of monoamine metabolism by the enzymes monoamine oxidase (MAO) A and B (Mukherjee and Yang 1997; Mukherjee and Yang 1999). McNamara *et al.* (2013) also investigated the effect of omega-3 rich or deficient diets on serotonin and 5-HIAA levels in a

range of brain regions (though not the brainstem) of rats that underwent a forced swim test. Contrary to this study, they found no significant differences between animals on the omega-3 deficient and rich diets. Brain stem monoamine (5-HT, NA and DA) levels were not significantly different between NS and untreated MS groups. However, 5-HIAA/5-HT and DOPAC/DA ratios, indicative of serotonin and dopamine turnover, were significantly higher and lower in untreated MS rats, respectively. Differences in expression of monoamine oxidases, receptors or transporters could account for these neurobiological findings in animals that displayed depressive and anxious behaviours. However, further investigation is required to elucidate such causations.

The role that the gut microbiota plays in regulating host health is now recognised as fundamental. Bidirectional communication between the gut microbiota and the brain, the microbiota-gut-brain axis, occurs through a number of pathways including the vagus nerve, the immune system, the neuroendocrine system and microbial metabolites (Sandhu *et al.* 2017). The microbiota-gut-brain axis provides the mechanistic pathways through which nutraceuticals can act to alter neurochemistry and ultimately behaviour. We have shown that administration of dietary fish oil altered the brain fatty acid profiles of rats, significantly increasing DHA relative abundance and decreasing omega-6: omega-3 ratios in the frontal cortex (Table 7.2.). Furthermore, treatment of early life stress with fish oil and fluoxetine significantly altered corticosterone and monoamine levels and turnover in the maternal separation animal model. To investigate possible pathways through which the dietary treatments (fish oil and fluoxetine) acted, we compared gut microbial composition and microbial metabolite (SCFA) concentrations in the different experimental groups.

Maternal separation has previously been shown to cause marked population-based alterations in faecal microbiota (O'Mahony *et al.* 2009). Here, compared to their non-separated counterparts, maternally separated animals were found to have significantly lower levels of *Lachnospiraceae* sp., *Christensenellaceae* as well as *Caldicoprobacteraceae* and *Caldicoprobacter*. These microbiota have previously been reported at reduced ratios in depressed individuals, indicating that they may be involved with psychiatric symptomatology (Naseribafrouei, 2014; Jiang *et al.* 2015; Pusceddu *et al.* 2015; Kelly *et al.* 2016; Tillmann *et al.* 2018). Interestingly, much of the microbiota found at significantly reduced ratios in MS-Con animals are involved in SCFA production. *Lachnospiraceae*_NC2004 group and *Ruminococcus* 2, both found at significantly lower ratios in MS-Con animals, were positively correlated to

total SCFA, acetate and propionate, and butyrate levels, respectively. These results add to previous studies reporting similar correlations between *Lachnospiraceae* and SCFA levels in mice (Robertson *et al.* 2017).

Dietary supplementation with fish oil caused the greatest change in faecal microbial composition. Animals with fish oil in their diet had a markedly lower Firmicutes: Bacteroidetes ratio. A high Firmicutes: Bacteroidetes ratio has been associated with high body mass index and obesity (Ley *et al.* 2005; Koliada *et al.* 2017) and irritable bowel syndrome (IBS; Rajilić–Stojanović *et al.* 2011; Liu *et al.* 2016). Furthermore, a robust clinical study found that a high Firmicutes: Bacteroidetes ratio was most commonly detected in IBS patients suffering from depression (Jeffery *et al.* 2012) and this microbial signature has since been reported in autistic children (Desbonnet *et al.* 2014) and depressed patients (Lin *et al.* 2017). Relative abundance of *Prevotellaceae* is another feature of microbial communities frequently recognised as a marker to distinguish between healthy and depressed groups. *Prevotellaceae* was the only microbial family found at a lower ratio in rats that received faecal microbiota transplants from depressed donors compared to those who received transplants from healthy donors (Kelly *et al.* 2016). Similarly, a study investigating the altered faecal microbiota composition in patients with MDD found significantly lower levels of *Prevotellaceae* compared to healthy controls (Jiang *et al.* 2015). Lower ratios of *Prevotellaceae* are also frequently cited as a characteristic of the gut microbial communities of people with Parkinson's disease and it has been suggested that this dysbiosis may lead to lower levels of mucin production and associated increases in gut permeability (Anderson *et al.* 2016; Dinan and Cryan 2017b). Direct links between *Prevotellaceae* abundance and neurochemistry have been found. Following traumatic brain injury, a decrease in the relative abundance of *Prevotellaceae* and a correlating increase in noradrenaline release and noradrenergic innervation of the cecum was reported in mice (Houlden *et al.* 2016). Higher abundances of *Prevotellaceae*/*Prevotella* are common in people with vegetarian and Mediterranean diets and they have been associated with dietary omega-3 PUFA (Pusceddu *et al.* 2015; Balfegó *et al.* 2016; Kelly *et al.* 2016; Dinan and Cryan 2017b). Here we found higher ratios of *Prevotellaceae* in both groups treated with fish oil, compared to MS-Con animals.

Fluoxetine has been shown to cause minor but significant compositional changes to gut microbiota (Cussotto *et al.* 2018; Lukić *et al.* 2019). It has been shown to inhibit the growth of Gram-positive bacteria, such as *Staphylococcus, Enterococcus* and *Lactobacillus*

rhamnosus, and the Gram-negative bacteria *Escherichia coli in-vitro* as well as significantly reducing the abundance of *Prevotella* and *Succinivibrio in-vivo* (Coban *et al.* 2009; Ayaz *et al.* 2015; Cussotto *et al.* 2018). In this study, the group administered fluoxetine harboured four genera at significantly higher ratios; *Neochlamydia, Lachnoclostridium, Acetitomaculum* and *Stenotrophomonas*. Three of these genera (*Neochlamydia, Acetitomaculum* and *Stenotrophomonas*) have not been associated with fluoxetine treatment previously. However, *Acetitomaculum* is an acetate-producing bacterium and its higher relative abundance in this treatment group positively correlates with the high concentrations of acetate also detected. Higher levels of this genus have been previously associated with a high-fat/Western diet in European children (De Filippo *et al.* 2010). In contrast, it was also found to notably decrease in mice upon antibiotic treatment (Hill *et al.* 2010). *Stenotrophomonas* is also a known carbohydrate fermenter (Auer *et al.* 2017). A recent study, investigating the effects of fluoxetine administration on the gut microbiota in mice, also reported a significant increase in *Lachnoclostridium* (Lyte *et al.* 2019). This genus has previously been associated with obesity (Amadou *et al.* 2016) and autism spectrum disorder (Luna *et al.* 2017) in humans, and the mouse study suggested that observed increases in this genus may indicate dysbiosis. However, *Lachnoclostridium* are SCFA producers and previous studies have found increases in this genus positively correlates with isovalerate (Lemaire *et al.* 2018).

Interestingly, we also show that microbial composition and SCFA concentrations correlate with significant differences between treatment groups. SCFA are metabolites produced by gut bacteria that have been shown to play a role in numerous biological systems, including energy and metabolism, immune function and neuroendocrine communication. They cross from the gut into the blood, allowing systemic circulation, and can pass through the blood brain barrier (Frost *et al.* 2014). Once in the brain they activate receptors and alter gene expression (Wei *et al.* 2015). While studies have concluded that SCFA influence gut motility and the gut epithelial barrier (Scheppach 1994; Fukuda *et al.* 2011), activate receptors related to appetite (e.g. G protein–coupled receptors; van de Wouw *et al.* 2017), and are found at different concentrations in healthy and depressed individuals (Skonieczna-Żydecka *et al.* 2018), many of the results from studies appear contradictory. For instance, SCFA have been found to stimulate peptide YY, which reduces appetite (Lach *et al.* 2018), but many studies have found higher levels of SCFA correlate with obesity (Zhou and Foster 2015). Kelly *et al.* (2016) reported faecal acetate and total SCFAs were increased in rats that received a

faecal microbiota transfer from depressed donors whereas Ohland *et al.* (2013) reported that mice fed a western-style diet displayed anxiety-like behaviour and had reduced total SCFA levels. In this study, maternally separated animals had significantly lower total SCFA, driven by reductions in acetate, propionate, iso-butyrate and iso-valerate. Fluoxetine treatment significantly increased total SCFA, principally driven by significant increases in acetate and propionate levels. SCFA are involved in neurotransmitter production (DeCastro *et al.* 2005) and have proven neuro-protecting abilities (Kidd and Schneider 2010). However, increased levels of SCFA, in particular propionic acid, have been linked to autism spectrum disorder (Cryan and Dinan 2012). Interestingly, fish oil treatment resulted in significantly reduced levels of butyrate, in this study. Similar results have been reported previously (Robertson *et al.* 2017). These results correlate with the changes seen in the microbiota compositions of these groups. Bacteria from the Bacteroidetes phyla mainly produce acetate and propionate, whereas butyrate is predominately synthesized by Firmicutes, which were significantly reduced in the MS-FO and MS-FX-FO groups. The beneficial effects of butyrate appear to be concentration dependant (Peng *et al.* 2007), with lower levels providing health benefits (Bourassa *et al.* 2016), while higher levels are linked to system dysfunction (Mariadason *et al.* 1999). Results from this study indicate that overall, a reduction in total SCFA is symptomatic of mental disorder, however, changes in the different SCFA should be considered individually as concentration dependant alterations could be significant. Furthermore, fluoxetine treatment resulted in neurobiological and behavioural changes associated with successful mitigation of depressive disorder, and this was accompanied by significant increases in caecal SCFA (acetate and propionate) levels.

A growing body of studies support the theory that gut-microbial composition can substantially affect central physiology and altered microbiota composition could be a direct cause of mood disorders like depression and anxiety. While symptoms for many patients remain intractable to standard pharmaceutical treatment, it is important to continue the search for new and adjunct therapies. Manipulation of gut microbial composition and their corresponding bioactive metabolites is a promising pathway for potential cognitive therapies. We investigated the effects of fish oil and fluoxetine, individually and as adjunct therapy for depression and anxiety. The study provides further support for the efficacy of the maternal separation model in rats as a suitable tool for studying these mood disorders. Furthermore, fish oil and fluoxetine treatment resulted in significant manipulation of important

neurobiological markers which translated to significant behavioural differences, representing a reduction in a depressive (fluoxetine) or anxious (fluoxetine, fish oil and the combination) state. Moreover, adjunct therapy of fish oil with fluoxetine appears to reduce the anti-depressant effects of the pharmaceutical compound. This diminished effect was accompanied by significant compositional changes in the gut microbiota and reduced caecal SCFA levels. Together these results suggest that modification of gut microbiota composition and production of SCFA could be an important mechanism through which fluoxetine provides anti-depressant effects. Although fish oil has not proved to enhance anti-depressant effects of fluoxetine in this study, its high levels of the important omega-3 PUFA, EPA and DHA, mean that it remains an important dietary component. However, the results here indicate the importance of considering pharmaceutical and dietary interactions during disease treatment.

Acknowledgements

We would like to gratefully acknowledge the funding of this work by the Department of Agriculture, Food and the Marine (DAFM), Ireland via the SMART FOOD project: 'Science Based 'Intelligent'/Functional and Medical Foods for Optimum Brain Health, Targeting Depression and Cognition' project (Ref No. 13/F/411). The lead author is funded by the Irish Research Council (IRC) and Biomarine Ingredients Ireland Ltd. via the IRC Enterprise Partnership Scheme. The other authors are supported in part by Science Foundation Ireland in the form of a centre grant (APC Microbiome Ireland Grant No. SFI/12/RC/2273); and the Sea Change Strategy, NutraMara programme (Grant-Aid Agreement No. MFFRI/07/01) with the support of the Marine Institute and DAFM in Ireland. We acknowledge the Teagasc Sequencing Facility, Dr Fiona Crispie, Dr Paul Cotter and Ms. Laura Finnegan for their technical assistance with the 16S rRNA MiSeq sequencing. Furthermore, we would like to acknowledge and thank Loreto Olavarria-Ramirez for her input into sample collection and analysis.

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SUPPLEMENTARY MATERIAL

Table S1. Diet macronutrient profiles (g 100 g^{-1} feed).

	Control	FO	FX	FX+FO
Casein	20.00	20.00	20.00	20.00
Maltodextrine	13.20	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00	10.00
Cellulose powder	5.00	5.00	5.00	5.00
L-Cystine	0.30	0.30	0.30	0.30
Vitamin Premix	1.00	1.00	1.00	1.00
Mineral and trace elements mix	3.50	3.50	3.50	3.50
Choline bitartrate	0.25	0.25	0.25	0.25
Corn starch*	39.75	39.75	39.74	39.74
Fluoxetine	-	-	0.02	0.02
Soybean oil*	7.00	-	7.00	-
Fish oil	-	7.00	-	7.00
LA	3.69	0.11	3.69	0.11
ALA	0.41	1.00	0.41	1.00
EPA	-	0.73	-	0.73
DHA	-	0.92	-	0.92
Omega - 6	3.69	0.32	3.69	0.32
Omega - 3	0.41	2.70	0.41	2.70

Table S2. Fatty acid profile of diets (g 100 g^{-1} oil).

	Control	**FO**	**FX**	**FX+FO**
SFA				
C14:0	0.28	6.98	0.28	6.98
C16:0	11.91	18.79	11.91	18.79
C17:0	0.14	-	0.14	-
C18:0	3.69	2.20	3.69	2.20
C20:0	0.43	0	0.43	0
C22:0	0	0	0	0
C24:0	0	0.30	0	0.30
Total SFA	16.45	28.26	16.45	28.26
MUFA				
C16:1 n-7	0.14	7.91	0.14	7.91
C18:1 n-7	0	4.24	0	4.24
C18:1 n-9	25.25	15.19	25.25	15.19
C20:1 n-9	0	0.83	0	0.83
C22:1 n-9	0	0.18	0	0.18
Total MUFA	25.39	28.36	25.39	28.36
PUFA				
n-6 PUFA				
C18:2 n-6	52.34	1.61	52.34	1.61
C20:2 n-6	0	0.14	0	0.14
C20:3 n-6	0	0.05	0	0.05
C20:4 n-6	0	1.83	0	1.83
C22:4 n-6	0	1.16	0	1.16
Total n-6 PUFA	52.34	4.80	52.34	4.80
n-3 PUFA				
C18:3 n-3	5.82	14.22	5.82	14.22
C20:5 n-3	0	10.37	0	10.37
C22:5 n-3	0	0.89	0	0.89
C22:6 n-3	0	13.10	0	13.10
Total n-3 PUFA	5.82	38.58	5.82	38.58
Total PUFA	58.16	43.38	58.16	43.38
n-6 : n-3	9.00	0.12	9.00	0.12

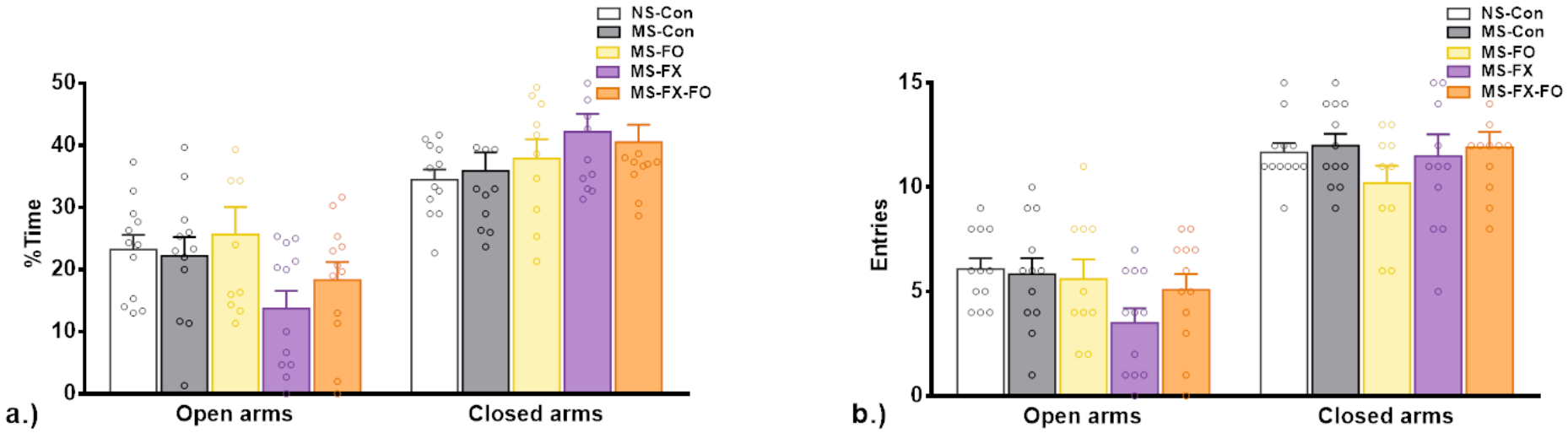

Figure S7.1. Plus maze test: the percentage of time spent in open and closed arms of the apparatus (a.) and the total number of entries into open and closed arms (b.). Values are means + SD for each experimental group (NS-Con, MS-Con, MS-FX and MS-FO-FX (*n*=12) and MS-FO (*n*=10)).

Chapter 8
General Discussion

GENERAL DISCUSSION

The effect of diet quality on human nutrition and health has long been recognised. Captured succinctly in Hippocrates famous quote, "*our food should be our medicine and our medicine should be our food*". In the modern era, more recently, there has been a realisation that the gut microbiota are heavily influenced by host nutrition and in turn play an important role in regulating host health and development. Fish and seafood are some of the most important dietary components for people worldwide and people who regularly consume fish have been found to have a reduced risk of all-cause mortality (Zhao *et al.,* 2016). As outlined in Chapter 3, fish provide proteins containing all the essential amino acids, polyunsaturated fatty acids (PUFA), calcium, iron and a range of other minerals and vitamins (Hamed *et al.,* 2015). Their global range means they are an accessible food source to many, often providing people's primary source of protein (Béné *et al.*, 2015). Furthermore, they can be considered a healthier option to terrestrial meat because their lipid fraction is highly unsaturated and often high in omega-3 PUFA. Indeed, they are the principal dietary source of omega-3 PUFA (Zhao *et al.*, 2016). However, a major concern of consuming fish is the possible presence of heavy metals and toxins that fish bioaccumulate throughout their life in the water. Mercury is a particular concern currently (EFSA, 2015) and this problem is only likely to increase in-line with increasing aquatic pollution levels. Monitoring and potentially extraction of heavy metals and other toxins from seafood are going to be an important consideration in the future.

The considerable health benefits associated with fish consumption mean that it should continue to be promoted and developed. However, as emphasised throughout this thesis, sustainable development is imperative. The seafood industry needs to develop within the recommendations of marine and fisheries experts, taking into consideration the impact on stocks as well as ecosystem services. The most obvious method of achieving this is through waste reduction and valorisation (Yan and Chen, 2015; Ferraro *et al.*, 2010). As outlined in the introduction, this will require innovative and novel processing methods to extract sought-after nutrients. These can then be used to produce functional dietary products for the human and animal healthcare markets.

In Chapter 5, we investigated the functional characteristics of fish protein hydrolysate powders produced using a range of enzymes, with a view to developing dietary protein supplements for beverage fortification. Nitrogen solubility was extremely high for all the

protein hydrolysate powders produced; a key characteristic required for powders used to fortify food and beverages. In addition, to the excellent essential amino acid profile of the powders makes them a very suitable ingredient for food and beverage fortification. Due to their health-promoting characteristics, research of fish protein hydrolysate (FPH) production continues to be an area of interest (Ishak and Sarbon, 2018; Dale *et al.*, 2019). The principal issue reported to hinder their use in functional foods is a fishy odour that is often associated with the powders (Shaviklo, 2015). Even in the grade A powders, which must contain no more than 0.75% fat, a fishy odour can develop after a period of storage (Jacobsen and Marinho, 2018). These factors highlight the importance of including shelf-life studies and sensory analysis in future work on production of functional foods and beverages fortified with FPH powders.

The protein hydrolysate powders produced in Chapter 5 contained 1.1 – 3.4% lipid fractions, which would need to be removed for the powders to be seriously considered as applicable functional ingredient options for human consumption. To minimise the lipid fraction, future production studies could assess the applicability of pre-treating the raw fish material prior to hydrolysis. Some studies have shown that pre-treatment with alcohols or acids help with reducing fishy or bitter flavours and odours in the end products, but these pre-treatments can also negatively affect hydrolysis and even other sensory properties such as colour (Benjakul *et al.*, 2018; Villamil *et al.*, 2017). Other deodorizing technologies that could possibly be tested include physical adsorption (e.g. activated carbon), microencapsulation or harnessing microbes that can degrade the volatile compounds causing the unacceptable odours (Wang *et al.*, 2019). Another option is to further develop the separation steps outlined in Chapter 5. This might include a process of physical separation and decanting; whereby proteins are heated after mincing to rupture the fat cells causing a portion of released fat to form a light liquid layer that can then be removed by decanting (Sathivel *et al.*, 2004). Following hydrolysis and the initial filtration, subsequent lipid separation by heating, centrifugation and decanting could be added, as well as possible further refined filtration to purify the soluble protein fraction.

In the final two chapters we carried out animal feeding trials to assess the effects of dietary fish protein and lipid supplementation on host growth, health and development, including changes to their gut microbiota. The newly developed company, Biomarine Ingredients Ireland Ltd., have successfully produced a number of products from blue whiting,

including fish oil, a soluble protein FPH containing 0.4% lipids (SPH) and, as a side-stream product, a partly-hydrolysed protein powder containing < 12% lipids (PHP). These SPH and PHP powders were used in Chapter 6 to supplement aquafeeds that were high in plant protein and low in fishmeal. In this study, we had hypothesised that the increased amino acid absorption rates and bioavailability, often reported for protein hydrolysates (Kristinsson and Rasco 2000; Manninen 2009), would provide enhanced nutrition, supplementing the deficiency of the plant proteins. At the end of the study Atlantic salmon parr that had been fed an 80% plant protein diet supplemented with PHP grew equally as well as parr that had been fed the positive control, 35% fishmeal, diet. A principle component of this study was the consideration of the effects of dietary changes on the host's gut microbiota. We found that the addition of dietary plant protein significantly altered the gut microbiota in Atlantic salmon parr, reducing alpha-diversity and relative abundance of lactic acid bacteria (LAB). Supplementation with SPH fish protein hydrolysate powder partly reduced these alterations in the gut microbial community. The reduction of LAB as a consequence of increased plant protein was opposite to reports from some previous studies that found LAB increased with high levels of dietary plant protein in adult Atlantic salmon (Hartviksen *et al.,* 2014: Schmidt, 2016). While its recognised that gut microbiota are important for converting nutrients into tangible growth and developmental changes, our knowledge and understanding the fish microbiome is in its infancy. Most studies investigating the gut microbiota of Atlantic salmon have focussed on adult fish in marine environments. There had been one recent study comparing the gut microbiota of Atlantic salmon parr sourced from hatcheries and from the wild (Webster *et al.,* 2018). From this it was reported that, overall, wild populations had considerably higher microbial diversity than hatchery populations. Therefore, the reduced alpha diversity of parr fed the high plant protein diets in this study may relate to a reduction in health. More research is urgently required to build a baseline understanding of the composition of salmon gut microbiota at all life stages so that the effects of dietary changes can be fully comprehended.

Although the fish on SPH supplemented diets had the most similar gut microbiota to the fish fed the fishmeal control diet, they did not grow as well. Interestingly, previous studies investigating FPH supplementation in aquafeeds have found that results are dose-dependent and negative effects can occur from higher levels of FPH inclusion (Espe *et al.* 1999; Hevrøy *et al.* 2005; Espe *et al.* 2012). In the study outlined in Chapter 6, PHP and SPH were

supplemented at the same level (10% of total diet). However, the molecular weights of the peptides and amino acids in the SPH diet were significantly smaller. It is conceivable that treatment with very low molecular weight proteins may have acted in a similar manner to a higher hydrolysate dose. However, why these higher doses negatively affect the growth outcome is still unclear. One difference that we found that could potentially explain the differing results in our study, was that branched chain amino acids were significantly higher in the plasma of fish on the PHP diet. These amino acids play important structural roles and act as an anabolic signal for protein synthesis (NRC 2011).

Both the PHP and SPH powders have a balanced amino acid profile that closely match the essential amino acid requirements of fish and humans. They are functional ingredients that hold high potential for the development of human healthcare products. Future studies should focus on their muscle accretion and sarcopenia prevention efficacy in sport nutrition and healthy aging. Thus far, we are aware of one study that has performed out such an investigation. Nygård *et al.* (2018) found that FPH supplementation at lower doses successfully stimulated muscle protein synthesis in elderly people.

Another line of research that could be pursued is the use of FPH supplements to enhance satiety. The satiating and ingesta reducing effects of fish protein have been reported by some as being comparatively superior to other sources (Uhe *et al.* 1992; Holt *et al.* 1995; Borzoei *et al.* 2006). However, other studies have not found significantly greater satiating effects from fish when compared to other sources (Pal and Ellis 2010; Boler *et al.* 2012). FPH may have stronger satiating abilities than whole fish. Previous research has found that FPH produced under specific hydrolysing conditions contained peptides that were secretagogue molecules (gastrin-like peptides) that could control digestive secretions (Guerard *et al.* 2001) and calcitonin gene related peptides that could regulate gastric acid secretion (Rousseau *et al.* 2001). An *in vitro* study showed FPH stimulating CCK-releasing activity in the STC-1 cell strain and was later tested, with positive results in a clinical trial for satiety and weight loss (Cudennec *et al.* 2008; Cudennec *et al.* 2012). Similar bioactive properties were demonstrated with FPH in rats, accompanied by a decrease in food intake as well as a decrease in body weight (Bougatef *et al.* 2010). It is important to recognise the possible conflicting uses of FPH to stimulate muscle accretion or invoke weight loss. Studies supporting both outcomes highlight the range of effects linked to different hydrolysing conditions and subsequently derived hydrolysates.

In Chapter 6, the promising results from this study warrant further investigation to confirm the effects and mechanisms of PHP supplementation in aquafeeds. The feeding trial was carried out under industry-relevant conditions, which meant that the results are robust towards natural variations in a farm flow-through system. However, future research that focusses on a mechanistic understanding of the dietary influences on the fish might benefit from scaling down the experimental design. Many peptides have bioactive qualities (Ryan *et al.* 2011) and have previously been shown to improve stress tolerance and immunity in fish (Ko *et al.* 2014; Khosravi *et al.* 2015; Gisbert *et al.* 2018). The possible bioactive qualities of these hydrolysates would be interesting to investigate. Other concepts to explore include the effect of FPH supplementation at different life stages as well as possibly providing the supplement in acute, rather than chronic, doses.

The final part of this thesis investigated the ability of fish oil treatment to prevent or reduce depressive and anxiety-like biological and behavioural symptoms. A recent systematic review of clinical trials focussed on the effects of omega-3 (EPA + DHA) fatty acid supplementation on brain health found that 19 out of 25 studies reported positive effects from omega-3 treatment (Derbyshire 2018). Only two of these studies focussed on mood and depression but both reported positive outcomes related to omega-3 treatment. Another review, including clinical and pre-clinical trials, focussing on DHA treatment, found that the effectiveness of DHA for treating depression disorders are still unclear (Ghasemi Fard *et al.* 2019). Similarly, a recent meta-analysis of 79 randomised control trials (112,059 participants) has presented results that suggest that increased levels of dietary long chain omega 3 fatty acids have little or no effect on all-cause mortality or a suite of cardiovascular illnesses and deaths (Abdelhamid *et al.*, 2018). Such reports from large review studies highlight the importance of continued investigations of health claims, despite any general long-standing beliefs. The results of our study do not definitively support nor disprove the efficacy of fish oil treatment to prevent or reduce depression, however the behavioural results were more positive for treatment of anxiety. The review by Ghasemi Fard *et al.* (2019) revealed that the most abundantly available and commonly studied fish oils are higher in EPA than DHA, often in a ratio of 18 : 12. However, they also report that studies have shown DHA to be the important fatty acid for treatment of heart and cardiovascular-related disease risk factors. We found that blue whiting (and boarfish and herring) fish oil is higher in DHA than EPA. A

higher ratio of DHA to EPA has also been reported previously for blue whiting and boarfish (Toppe *et al.* 2007; Gormley 2015; Mika *et al.* 2016). While reports are more variable for herring, possibly due to differences between size classes (Toppe *et al.* 2007) and variation in diet. It would be interesting to test and/ or compare the efficacy of high DHA fish oils with high EPA fish oils for treating risk factors of heart disease.

Some noteworthy differences in the gut microbiota composition were found between the different treatment groups in this study. Reduced levels of specific families of microbiota (*Lachnospiraceae, Christensenellaceae,* etc.) in the maternally separated group were in-line with results previously reported and possibly act as indicators of depression (Naseribafrouei, 2014; Jiang *et al.* 2015; Pusceddu *et al.* 2015; Kelly *et al.* 2016; Tillmann *et al.* 2018). Such knowledge allows for development of directed treatment. Similarly, fish oil supplementation created significant reductions in the Firmicutes: Bacteroidetes ratio and increased levels of *Prevotellaceae.* Both these factors are characteristics of host gut microbiota associated with positive health benefits. Thus, such results lend further support for the positive benefits of fish oil supplementation.

Another interesting outcome of the fish oil study was the effect fish oil treatment had on caecal butyrate levels. Developing research suggests short chain fatty acids are important molecules in a number of signalling pathways (Cani and Knauf 2016). However, a significant amount of studies published present contradictory results (Sakata 2019) due to variations in methodologies. Furthermore, it may be that the actions of SCFA and the effects of high or low concentrations are complex, unique to each metabolite and do not follow a simplistic understanding such as more equals better. The butyrate paradox lends support for such a proposal (Peng *et al.* 2007; Bourassa *et al.* 2016). Short chain fatty acids appear to be a way in which dietary intervention can influence many different biological systems. Studies have reported the separate effects of SCFA and fish oils on the neuroendocrine and immune systems but there is little research, thus far, on their interactions (Galli and Calder 2009; Grosso *et al.* 2014; El Aidy *et al.* 2015). Since fish oils are one of the longest standing and most popular dietary supplements this is a worthy area of research to develop in the future (Kirk *et al.*, 1998; NCCIH, 2012).

Throughout this thesis, the influence of the gut microbiota has been considered. Over the past number of decades the gut microbiota has gone from being thought of as a largely inert and homeostatic array of microorganisms which had little relevance in disease, to a

virtual organ that plays an essential role in human and animal health through its effects on host immunity and metabolic health (Blaser 2014; Robertson *et al.* 2017). Research on the fish gut microbiota is a growing field, largely developed around the aquaculture industry. There is significantly less research carried out on the gut microbiota of wild fish (Egerton *et al.* 2018). However, an understanding of the microbial state of fish in their natural environment is imperative to guide future work. Furthermore, knowledge of a species' or populations' microbiome could help to indicate differences important to fisheries scientists and marine ecologists in terms of host health, population interactions and ecosystem services, to name but a few.

I conclude by recognising that the work presented within this thesis is of a diverse nature. It spans the steps of biotechnology; beginning by gaining an understanding of the raw material, moving on to develop a product and finally testing the efficacy of such products for improving health and nutrition. Interdisciplinary research requires a diverse understanding of different areas which presents challenges for the researchers. In addition, it can be limited in the practical depth of investigation. However, our world is an interconnected one, and the challenges we face will not be overcome without research that crosses disciplines and explores the big picture as well as the small. This body of work has provided advancements in multiple fields of research and concludes by providing numerous opportunities for the development of future work leading to in-depth research on these fascinating species for their efficacious application in the generation of improved food ingredients for animal and human health.

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Acknowledgements

Acknowledgments

My sincere thanks to my supervisors, Paul Ross, Catherine Stanton and Sarah Culloty, who provided me with this career forming opportunity. Their belief and support from the point of applying for funding continued, unwavering and has allowed me to pursue a Ph.D. that followed my interests and built on my capabilities. I recognise the efforts that had to be made on all of your parts to provide me with the resources to complete this Ph.D., and I am immensely grateful. I must also thank University College Cork, APC Microbiome Ireland, Teagasc and the Marine Institute. These organisations and the incredible staff within them supported me throughout my three and half years of study.

This next section is dedicated to all the incredible people who contributed to Chapter 6, and all the related studies which I hope will follow on from it. Firstly, a special thank you to Phil McGinnity, a generous man and a wonderful mentor. Thank you for taking me under your wing and teaching me a little bit about salmon. Your knowledge, guidance and friendship were gratefully received. I'll be forever thankful for your good humour, patience and support with the ethics debacle. Thanks to Pete McGovern; all the team in Newport, especially Fintan and Liz; the amazing Fish Crew - Fergus, Ivan, Grace, Kiz and Clodagh, my volunteers Victoria and Kieran; Niall and the NUIG gang. All of you gave your time so generously. I owe a lot of favours. The summer of 2018 would not have been barrable without all of you. You brought a lot of craic to Salmon Springs and all worked so hard as well!! Finally, of course, Alex – you were great. I didn't know what I'd taken on when I started this project and I am 100% sure it would not have been a success without you. I learnt so much from you and you were there with support and answers whenever I needed you. Thanks.

Thanks to all my friends and colleagues in Teagasc, especially all the researchers, past and present, of APC1 and 2. I've had a great time being part of this massive family of nerds. Ivan and Daragh – the inseparable pair. Thanks for always bringing the craic and for keeping me real. Looking forward to many more laughs with you two. Ferg, Jules, Cat thanks for all the chats, laughs, nights out and procrastinating coffees. You guys were my besties from the start, so thanks for your friendship. Ferg, a special thanks to the massive ball of positivity that

you are. It's been a sad 2019 without you in the office and I don't think I'll ever have a colleague or friend who brings a smile to everyone's face as quickly as you do.

As it is for most things in my life, I could not have completed this PhD without the love and support of my friends and family. People say that a PhD is a great feat for a person to accomplish but in reality, it is the achievement of many, and it certainly takes a degree of selfishness, when one's mind and energy is focussed on a single goal. Thanks to my nearest and dearest for sticking with me through it all. To Noreen, my Claire's (O'Farrell, Canning), Aoife thanks for wiping away the tears and for the constant encouragement. I love you all.

Eric and Deefer, I've made it to the end with you two. You've been amazing. Thanks for all the company, cuddles, hot dinners, long walks and happy distractions. You two put it all in perspective and I'm very lucky to have you in my life. *#Blessed*.

Most importantly, thank you to my parents and siblings. Polly, it's amazing having you home (never leave). You're always there with honest, sound advice. I hope you know how much I appreciate and adore you. Mum and Dad, you've supported me in every life choice, every adventure, migration, academic and career decision, regardless of the practicality of them. I'm so fortunate to have such open-minded, selfless parents. I'm so thankful for your advice and guidance. You're both an inspiration to me in so many ways.

The final line must go to my Grandad, Paddy. If only I'd listened to you, I could have saved myself ten years! Who knew that head-strong hippie would end up counting salmon in Fermoy and not turtles in Madagascar! You blazed this trail. You tried to make it easy for me, but I guess we all need to find our own way. Regardless, I feel proud to be following, in some way, in your footsteps. This is for you, a champion of science, business, progress and development. You are truly missed.